Biological Control of Wood Decay in Ground Contact Timbers

Heather L. Brown

This thesis is presented to the University of Abertay Dundee in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy.

Scottish Institute for Wood Technology,
School of Science and Engineering,
University of Abertay Dundee.

June 2002

I certify that this is the true and accurate version of the thesis approved by the examiners.

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(Director of Studies)

This manuscript is dedicated to Eilidh Darroch

and the Don't Care Bear.

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Abstract

Wood placed in ground contact situations is susceptible to attack by a wide range of soil micro-organisms which degrade the wood structure, causing significant deterioration of the mechanical properties of the timber such as strength and elasticity. Chemical preservatives such as CCA (copper-chrome-arsenic) and CCB (copper-chrome-boron) are currently used to prevent the decay of wood exposed to biodeteriogens. However, increasing concerns regarding the long-term environmental effects of the toxic chemicals applied to ground contact timbers has resulted in the investigation and development of a number of alternatives to chemical preservation.

This project examined the use of biological control as a means of protecting ground contact timbers from fungal decay. *Trichoderma* spp. are soil-inhabiting fungi with broad-spectrum antagonistic properties, and have been extensively investigated as potential biocontrol agents in agriculture and forestry.

A small-scale wood-based screening system was developed for the rapid assessment of biocontrol potential. The screening system used non-sterile soil as a test medium to provide a natural environment for the evaluation of potential biocontrol agents. A number of *Trichoderma* isolates were tested using the developed system, and one *Trichoderma viride* isolate, (T60) demonstrated an initial protective effect against non-sterile soil. This isolate has previously been shown to be totally effective against basidiomycete decay fungi. The isolate was therefore selected as a potential biocontrol agent for ground contact timber, and a field trial was designed to assess the isolate on a larger scale.

Development of the field trial included the formulation of an appropriate delivery system. The biocontrol agent was applied to wood as a fungal spore suspension prepared in water. Wood was pressure impregnated with spores using a pilot preservation plant, integrating biological control strategies with current industry processes. Spore viability was retained, as indicated by the germination of *Trichoderma* spores on the wood surface.

Field trial and fungal cellar testing of the biocontrol agent on ground contact was based on European Standard test methods for chemical wood preservatives. T60-treated stakes, CCA-treated stakes and untreated control stakes of 2 softwood species were uplifted after 9 and 18 months exposure to soil. Recommended subjective assessments were supplemented with biological examinations of decay. Results indicated partial protection of T60-treated wood. However, the study highlighted the influence of test system and wood species on the activity and behaviour of the applied biocontrol agent.

To assess the distribution of the biocontrol agent in wood following the novel application procedure, a molecular-based method for the specific detection and quantification of T60 in treated wood was investigated. DNA was extracted from wood and amplified using the polymerase chain reaction (PCR). A random primer was used to detect T60 DNA in extracted material, and image analysis software was utilised to assess the relative abundance of amplified DNA indicating a non-uniform distribution of detected spores.

In summary, results from the field and fungal cellar testing of a selected biocontrol isolate showed a variation in the degree and constancy of protection against wood decay fungi. Standard testing guidelines were used as a basis for a field trial, with modifications designed to provide a more comprehensive assessment of biological control in ground contact timbers. The research presented in this thesis highlights the importance of field testing with regard to the biological control of wood decay, and indicates the influence of environmental factors on the overall performance of fungal biocontrol agents.

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Abbreviations

bp	base pairs
BSA	bovine serum albumin
[10 x]	concentration (e.g. 10 times normal)
CCA	copper-chrome-arsenic
CCB	copper-chrome-boron
°C	degrees Centigrade
DNA	deoxyribonucleic acid
dH_2O	distilled water
dNTP's	deoxynucleotide triphosphates
EDTA	diaminoethanetetra-acetic acid disodium salt
ELISA	enzyme-linked immunosorbent assay
FSP	fibre saturation point
g	grams
g	measure of centrifugal force
HCl	hydrochloric acid
ITS	internal transcribed spacer
L	litre
LN_2	liquid nitrogen
LNM	Low Nutrient Media
MEA	malt extract agar
µg	micrograms
µl	microlitres
mA	milliAmps
mg	milligrams
ml	millilitres
mm	millimetres
mM	milliMolar
M	Molar
NaCl	sodium chloride
ng	nanograms
nm	nanometres
N	normal (concentration of acids/alkalis)
%	per cent
PCR	Polymerase Chain Reaction
ppm	parts per million
RAPD	Randomly Amplified Polymorphic DNA
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulphate
Taq	*Taq* DNA polymerase
TE	Tris-EDTA
TSM	*Trichoderma* Selective Media
T60	*Trichoderma viride* isolate T60
U	Unit (universal measure of enzyme)
UV	ultraviolet light
v/v	volume per volume
WHC	Water Holding Capacity
w/v	weight per volume
w/w	weight per weight

Chapter 1

Introduction

1.1 Uses of wood

Wood is an important natural renewable resource which can be easily worked and has a multitude of uses. In its simplest application, the energy stored in wood is used to produce heat for cooking or for industrial process and therefore world-wide utilisation of wood is primarily for fuel. Wood serves as an important structural resource, providing a renewable material with high strength per unit weight (i.e. on a weight for weight basis, wood is stronger than steel). In many countries, wood is the primary construction material (Morrell and Gartner, 1998) and from an ecological viewpoint, in contrast with the finite reserves of iron and plastic bases available, wood stocks, with careful management are renewable. In fact, the world production of roundwood is of the same order of magnitude (by weight) as the production of steel and iron (Schniewind, 1989).

The qualities which make wood such an attractive and versatile material are related to the structure of the growing tree. The highly ordered microfibrils and crystalline structure of cellulose combine to give a high tensile strength to mass ratio, which reflects the need for a material that can support a leafy canopy without adding excessive weight to the support structure (Morrell and Gartner, 1998). The high void volume of wood makes it an excellent thermal insulator in comparison with either steel or concrete; the low levels of metals means that wood is an excellent insulator for electricity - wood is selected for distribution poles carrying overhead electrical lines because of its unique combination of high strength per unit weight ratios and low electrical conductivity. The permeable nature of wood as a result of both its structure and its structural components is a major influence on processes such as pulping, gluing and impregnation with chemicals, and the elasticity and strength of wood also makes it ideal for shock absorption.

There are, however, a number of characteristics of wood which are disadvantageous to the user including susceptibility to damage, either by decay, fire and chemical or mechanical breakdown. Wood is anisotropic, i.e. it swells and shrinks during wetting and drying, and these dimensional changes will occur more in the radial and tangential directions then longitudinally (Morrell and Gartner, 1998); it is also hygroscopic, meaning that it will absorb or lose water in relation to the temperature and humidity of the surrounding air (Morrell and Gartner, 1998). However, modern technology has overcome some of these inherent disadvantages of wood. For example, modern fire retardants limit the extent to which wood can be destroyed by fire (King, 1981).

1.2 Structure of Wood

Wood is a natural composite material, consisting of three major structural polymers i.e. cellulose, hemicellulose and lignin (Core *et al.*, 1979). These polymers comprise around 90-98% of the wood mass - the remainder is made up of wood extractives including phenolic compounds, lipids and proteins (Morrell and Gartner, 1998).

The framework substance of wood is cellulose, which contains repeating units of β 1-4 linked D-glucose. Individual chains may contain as many as two thousand glucose units and be up to five millimetres long. Cellulose chains are oriented at parts into crystalline structures referred to as microfibrils however other regions remain amorphous. Cellulose microfibrils form the skeleton of the wood cell wall and make up approximately 40-50% of the wood on a dry weight basis. The highly ordered nature of the microfibrils is what gives wood its high tensile strength (Morrell and Gartner, 1998) as well as providing the elasticity which allows the growing tree to withstand environmental stresses such as the wind.

Hemicellulose and lignin constitute the remainder of the wood cell wall structure and the amount of each compound present is dependent on the wood species and type, for instance softwoods generally have a higher lignin content than hardwoods but less hemicellulose (Core *et al.*, 1979). Hemicelluloses are a heterogeneous class of polymers containing sugars such as glucose, mannose and xylose. Although it has been believed that hemicelluloses do not contribute substantially to the structural properties of wood, recent studies suggest that it is an integral component of the lignocellulosic matrix and may play a role in the resistance of wood to impact (Morrell and Gartner, 1998). It is thought that hemicellulose acts as a coupling agent between the hydrophilic cellulose microfibrils and the hydrophobic lignin matrix. If this is the case, in the absence of this polymer water films could develop along the microfibril/matrix interface, decreasing the strength of the wood. A number of biodeterioration studies have shown that hemicelluloses are among the first of the wood polymers to be degraded, suggesting that their utilisation may represent a first key step in the decay process (Green and Highley, 1995).

Lignin polymers have high molecular weights and are highly branched, composed of phenyl-propanol units with numerous types of linkage between individual units. Although the nature of the repeating units and many of the linkages have been investigated, only a few lignin structures have been elucidated and in general their structures remain largely unknown (Morrell and Gartner, 1998). Lignins provide rigidity to the wood by encasing the cellulose, and their viscoelastic nature is vital to the structure of a growing tree. Also, by coating and protecting the cellulose microfibrils, lignin is believed to improve the durability of wood against microbial attack - while many organisms have evolved the ability to utilise cellulose or hemicellulose, relatively few types can decompose lignin effectively (Eaton and Hale, 1993).

Extractives and other substances, although in relatively short supply, are also important components of wood. Despite the relatively low amounts of these substances, they will still have a significant effect on the rate of decay of wood. Materials present in the sapwood such as pectin, starch and low molecular weight carbohydrates are thought to be especially important as initial carbon sources for the establishment of micro-organisms in wood (Nayagam, 1987; Breuil, 1998). Nitrogenous materials, present in small amounts in wood, are essential to the growth and activity of wood decay micro-organisms as they provide an extraneous nitrogen source for the production of wood-degrading enzymes. Heartwood extractives are generally more toxic to invading micro-organisms than those materials found in sapwood and can enhance durability and decay resistance properties of some wood species, such as cedar (Morrell and Gartner, 1998). The biochemical nature of these compounds, for example waxes, can also have an effect on moisture movement within wood.

1.3 Structure of the wood cell wall

There are multiple layers to the wood cell wall - the primary cell wall and three layers of the secondary cell wall (see Figure 1.1). On the outside of the primary cell wall is the middle lamella, an intercellular attachment between the primary cell walls of adjacent cells. The primary cell wall and the middle lamella regions have a high lignin content and very little cellulose. These two layers are generally considered together and compose the smallest area of the cell wall (Morrell and Gartner, 1998).

The secondary cell wall is composed of three distinct layers, referred to as the S_1, S_2 and S_3 layers in the order of their formation (S_1 adjacent to the primary cell wall and S_3 lining the cell lumen). These three layers differ in their composition in terms of relative amounts of cellulose, and S_2 has the highest proportion of this polymer. The orientation of the cellulose microfibrils within the sections of the secondary cell wall

layers can distinguish between them - the helical arrangement of the microfibrils in the S_1 and S_3 layers can be seen to follow an almost equatorial angle (50 °-75° in S_1 and 60°-80° in S_3) while in the S_2 layer, the orientation of the helices is extremely steep, around 10°-30° to the cell axis (Wilson and White, 1986). The higher cellulose content of the S_2 layer and the placement of those microfibrils is generally considered to be responsible for much of the modulus of elasticity of wood removed from trees. The S_3 layer is more lignified in softwoods, and it has been proposed that this allows the S_3 layer to act as a barrier to biodegradation (Daniel and Nilsson, 1998).

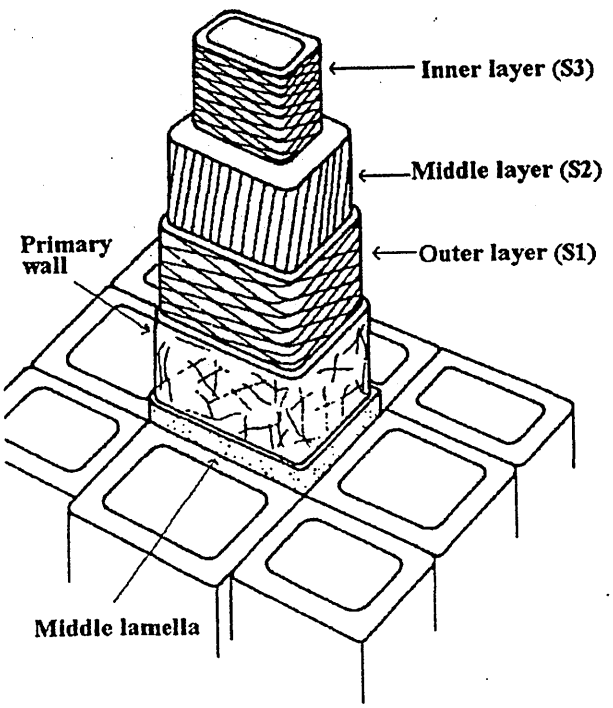


Figure 1.1: The structure of the wood cell wall (taken from Desch and Dinwoodie, 1996)

1.4 Structural differences between softwoods and hardwoods

Timber is generally divided into two groups: the hardwoods such as ash, beech, walnut or oak; and the softwoods (Levy and Dickinson, 1981). Commercially important species of softwood are those such as pine, spruce and Douglas fir. The terminology hardwood and softwood does not refer to the hardness of the wood, but rather to the family/order of the tree species, i.e. hardwoods are angiosperms and softwoods are gymnosperms. There are significant differences between the two wood types at both a structural and a chemical level (Jane, 1970), and these differences are largely responsible for the greater decay susceptibility of some hardwoods.

1.4.1 Softwoods

Softwoods are comprised principally of vertically oriented cells called tracheids, which in the living tree are responsible for support and fluid conduction. This cell type accounts for around 90% of the total cell volume of the wood (Wilkinson, 1979); the radial system of softwoods contains ray parenchyma cells and may contain ray tracheids and resin canals (Morrell and Gartner, 1998). Some species also have axial ray parenchyma tissues but this generally accounts for only a small proportion of the wood (Jane, 1970). The ray cells are where the food reserves are stored in the sapwood of the living tree, e.g. starch, sugars, amino acids etc.

Tracheids are hollow, square to rectangular when viewed in cross-section, and have closed, tapering, overlapping ends. Although cell types and sizes vary depending on the tree species, tracheids are commonly one to several millimetres in length and have a "diameter" of around 10-60μm. Flow of liquid from one tracheid to another in the living tree occurs via small openings in the cell wall called pits (Wilkinson, 1979). Situated across some adjoining pits is a specialised membrane, the torus, which can move as the sap pressure rises or falls in the adjacent fibres. This membrane is

composed primarily from cellulose and pectin and is usually permeable in sapwood pits. The torus is secured to the middle lamella by a complex of filament-like strands called the margo (Morrell and Gartner, 1998). In the heartwood, pit membranes tend to be impermeable due to the build up of materials produced during the conversion of sapwood to heartwood (Morrell and Gartner, 1998; Morrell and Morris, 2002). These deposits are water insoluble and so the process compounds the difficulties of achieving complete and even preservative penetration into heartwood.

Pits can be simple, semi-bordered or bordered in nature and generally occur in pairs. If a pit occurs in a parenchyma cell wall, there is no overhanging border and this is a simple pit (Core *et al.*, 1979). In a "prosenchyma" cell such as a fibre or tracheid, the pit chamber is usually enclosed with a border (except at the aperture) and this is a bordered pit. Half-bordered pits occur when one of the pits in the pair is simple (i.e. from a parenchyma cell) and adjoins to a bordered pit in the tracheid or fibre cell wall. A major physiological difference between softwoods and hardwoods can be seen in the bordered pit pairs - a torus is often present in the centre of the porous membrane of pits in conifers whereas in hardwoods the pits are simply holes (Core *et al.*, 1979).

In most species of timber, sapwood is easily penetrated by chemical preservatives. This is because, in the growing tree, the sapwood contains the cells responsible for the conduction of fluids. There are some wood species, however, such as eastern hemlock (*Tsuga conadensis*) and many firs (*Abies* spp.) and spruces (*Picea* spp.) in which impregnation of the sapwood with chemicals can only be achieved to a limited extent. Timber types displaying this characteristic are referred to as refractory timbers. The reason for limited penetration is due to aspiration of the pit membrane in softwood bordered pit pairs. The torus moves to one side during drying and effectively seals off the pit as a means of fluid conduction (Core *et al.*, 1979). Aspirated (closed)

pits are considered to be a major factor in the refractory nature of certain softwoods by reducing the natural permeability of the wood (Green, 1988).

Several methods have been attempted to improve penetration in refractory wood species, including incising where a series of radial cuts are made into the surface of the wood; altering the pre-treatment drying regime (Morrell and Morris, 2002); steaming in which wood is subjected to high-pressure steam in a bid to re-open the pit membranes; sap-displacement treatment where wood is treated whilst still "green", thereby avoiding aspiration (Hainey, 1992);and wet storage (ponding), where timber is retained in a wet state prior to treatment. This last method has several purposes – retaining high moisture content reduces the occurrence and effects of aspiration, and the anaerobic conditions reduce most microbial activity. However, some bacteria may still colonise the stored wood and degrade the pit membranes, increasing permeability of the timber (Wilkinson, 1979; Green, 1988). Other approaches to improving preservative penetration in refractory wood species include altering the viscosity of the carrier solution (Morrell and Morris, 2002).

1.4.2 Hardwoods

The microscopic structure of hardwoods is usually characterised by wood of a more heterogeneous configuration (Morrell and Gartner, 1998). Structural support for the tree is mainly from the fibres. These are the hardwood equivalent to the softwood tracheids and like tracheids, are the principal cell type. The physiological differences between the fibres and tracheids are that fibres are typically shorter in length with thicker cell walls and fewer pits. This is due to the fact that although some fluid conduction takes place via fibres, the majority is achieved via the vessel elements - these are open-ended cells which connect end to end to form conduit-like structures called vessels. These cells are much larger than fibres, ranging in width from 20-200

μm and varying greatly in length. Hardwood storage tissue consists of parenchyma cells in both vertical and horizontal orientations, made up from longitudinal parenchyma and ray parenchyma cells respectively.

At the level of chemical composition, the cellulose contents of softwoods and hardwoods are similar, around 40-50% of the total wood dry weight. However, the lignin content of softwoods is generally significantly higher than hardwoods - 25-34% and 17-24% respectively. The composition of the lignin is also different, with guaiacyl units being the primary lignin building block in softwoods whereas both syringyl and guaiacyl lignins are present in hardwoods (Green, 1988; Daniel and Nilsson, 1998).

1.5 Moisture content of wood

Moisture content is one of the most important factors influencing the service performance of wood. The amount of water present not only affects the strength and mode of failure of in-service timber, but also its dimensions and its susceptibility to decay. For wood to perform well and display maximum strength and elasticity properties, the moisture content must be reduced to at least 12% of its oven dry mass (Desch and Dinwoodie, 1996).

When a tree is felled, moisture is present in two forms: free water and bound or fixed water. Free water refers to the water present within the cell cavities, i.e. the lumen. It is not held in the wood and the removal of this water during drying has no effect whatsoever on either the mechanical performance of the wood or its dimensions (Desch and Dinwoodie, 1996). Fixed water is present in the wood cell walls, bound to the hydroxyl groups of the cellulose microfibril sheath by hydrogen bonds. The cell walls of most timbers can hold around 25-30% of their dry mass (Eaton and Hale, 1993; Desch and Dinwoodie, 1996). When all the free water has been removed and the maximum amount of bound water is present in the wood cell walls, the wood is

described as being at the fibre saturation point (FSP). The fibre saturation point is critical in decay of timber as the presence of free water is essential for fungal growth and development and also provides a medium for the diffusion of microbial enzymes (Morrell and Gartner, 1998). Decay fungi can only cause serious damage when wood is above the fibre saturation point (Green, 1988). Therefore, reducing the moisture content of wood below the fibre saturation point can greatly inhibit the degradative activities of wood decay fungi (Sinclair, 1995). In addition to this, as bound water is removed from within the cell walls the cellulose microfibrils move closer together, increasing the strength of the wood cell wall and hence the overall strength of the timber (Desch and Dinwoodie, 1996). An indication of the typical moisture tolerance of certain wood-inhabiting micro-organisms is shown in Figure 1.2.

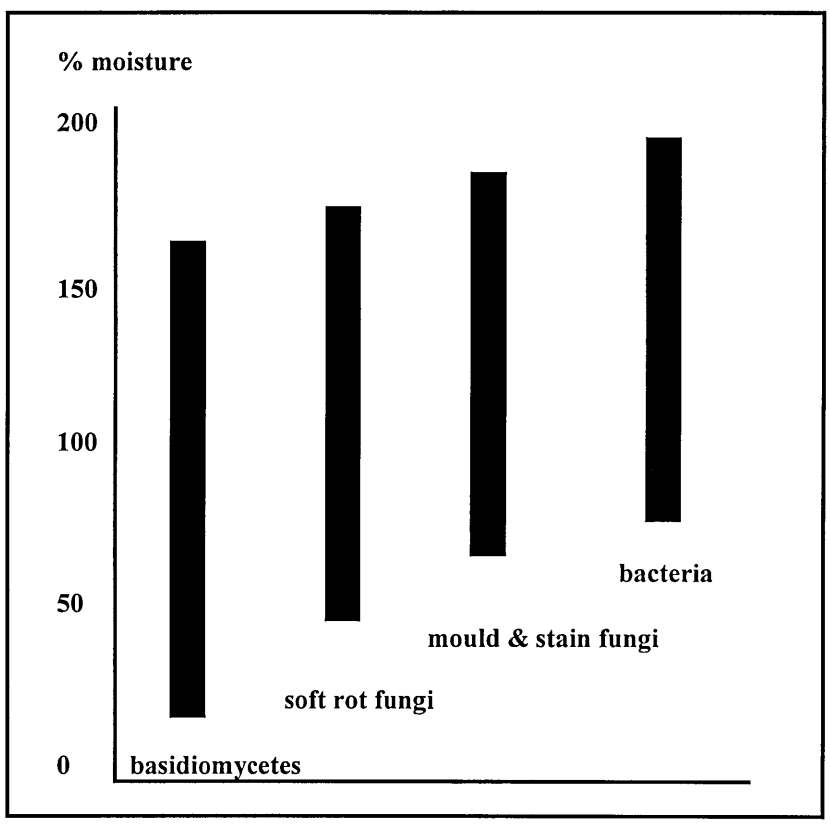


Figure 1.2: Moisture ranges for the colonisation of wood by micro-organisms (adapted from King, 1981)

Excessive amounts of water in timber can lead to waterlogging, where the lumens of the wood cell wall fill with water. This limits the amount of oxygen available for aerobic organisms, retarding microbial attack, and protecting lumber prior to service in this manner is referred to as "ponding" (Hainey, 1992). Decay is not completely prevented as anaerobic bacteria can eventually cause substantial damage to the wood (Eaton and Hale, 1993); moderate pectinolytic activity under controlled circumstances can, however, serve to increase the permeability and preservative retention of wood.

1.6 Nitrogen content of wood

Nitrogen is an essential constituent required for the synthesis of a wide range of compounds, e.g. structural proteins, nucleotide bases, enzymes etc., making it a critical element in all biological systems (Mowe, 1983). The nitrogen present in wood occurs in many different forms. The bulk of this nitrogen is in the form of cell wall protein and is not immediately available to colonising organisms, which makes nitrogen availability a limiting factor for fungi colonising wood (Breuil, 1998). Nutrients such as protein are present in the sapwood of freshly sawn lumber (Breuil, 1998), and other structures of the wood cell and dead cells also contain a proportion of nitrogenous compounds. The overall nitrogen content of wood ranges from 0.03% - 0.10% (w/w) with carbon to nitrogen ratios (C:N) varying between 350-500:1 (Cowling and Merrill, 1966). Soluble nitrogen is present in marginal amounts in sapwood, and the composition of soluble nitrogenous compounds has been investigated by a number of authors (Kirk, 1973; Nayagam, 1987). Free amino acids were found to be present in sapwood in much higher concentrations than the minimal amounts of the same compounds found in heartwood (Nayagam, 1987). Studies have also shown that the most soluble nitrogen occurs as amino acids rather than nitrates or ammonium compounds (Nayagam, 1987). The effects of added nitrogenous material

in the rate of wood decay have been known for a number of years (Savory, 1954). It has been noted that the type of nitrogen source as well as concentration are important factors influencing the decay of wood by soft rot fungi (Butcher and Drysdale, 1974), and it has been suggested that the ratio of carbon to nitrogen is a more important determinant in the decay of wood by fungi than absolute amounts of nitrogen (Mowe, 1983).

The importance of soluble nitrogenous compounds is not restricted to their concentration or composition. King, Oxley and Long (1976) demonstrated that these materials migrated and accumulated at evaporative faces of wood during drying, a process referred to as redistribution of soluble nutrients.

Soluble nitrogen may not be the only source of extraneous nitrogen available to wood-colonising micro-organisms. Under certain conditions the nitrogen requirements of a wood-colonising organism may be met by sources outwith the wood itself. For example, fungi present in wood in ground contact may assimilate nitrogen from the soil. Three different mechanisms have been proposed for the facilitation of increased nitrogen in ground contact timber: wick movement as demonstrated by Baines and Levy (1979); nitrogen fixation through bacterial action (Sharpe and Millbank, 1973; Levy *et al.*, 1974); and microbial transfer (King *et al.*, 1981). All three of the postulated processes may have significant roles in the decay of timber in ground contact situations by increasing nitrogen contents of wood (Mowe, 1983).

Availability of nitrogen is another major factor affecting the initial rate of decay of wood by fungi. During drying of freshly felled timber, redistribution of soluble nitrogen and other nutrients occurs as compounds are carried to the surface of the wood and deposited in concentrations five to ten times those of sub-surface wood (Green, 1988) as the moisture evaporates. The presence of such nutrients, referred to

as redistributed soluble nutrients (RSN), is thought to have a significant effect on the pattern of decay observed during the soil burial of wood blocks (King, Oxley and Long, 1976). The presence of RSN results in increased nutrient availability on the wood surface, and it has been suggested that this provides a stimulus to active invasion (Mowe, 1983; Waite and King, 1979), particularly by soft rot fungi, which generally require more extraneous nitrogen in order to produce wood-degrading enzymes than most other wood decay micro-organisms (Daniel and Nilsson, 1998).

1.7 Biological damage

Although wood does not corrode as such, it is susceptible to biological damage. This can be in the form of marine borer damage, insect damage and microbial decay (decomposition or biodeterioration). In temperate climates micro-organisms are the primary agents of wood decomposition. Wood can be decomposed by micro-organisms either in the form of a living tree or after it has been felled. When it is decomposed in the standing form, the organisms involved are disease-causing parasites, and this is classified as plant or forest pathology. From the viewpoint of the wood user, the most important micro-organisms are generally those which colonise wood after it is felled (King, 1981). Fungi require a nutrient source, appropriate temperature, oxygen and water in order to colonise wood (Wilkinson, 1979). A good example of how these factors interact can be seen in ground contact timber. The wood above ground is rarely wet long enough for fungi to become established and low oxygen levels deep below the ground also retard growth. It is the region of the stake around ground level that has optimal conditions for fungal growth and decay to take place (Wilkinson, 1979).

Susceptibility of wood to decay depends on a number of factors including the structural and chemical composition (i.e. nutrients) of the wood - this differs depending on the wood species and type (softwood, hardwood, sapwood and heartwood) as does

the colonisation sequence of wood by micro-organisms (Eaton and Hale, 1993). Other factors influencing decay susceptibility are density of the wood; orientation of sawn wood (which of the longitudinal, tangential or radial faces of the timber are most accessible to potential colonisation by decay fungi); moisture content; redistribution of soluble nutrients; ease of microbial infection; pH and temperature.

1.8 Ground contact timber

Wood in service is categorised into hazard classes according to its use, situation and the associated risk of decay. These hazard classes are outlined in European Standard EN 335 "Hazard classes of wood and wood-based products against biological attack" (CEN, 1992) and range from a minimal decay risk of 1, a situation in which the wood is under cover, fully protected from the weather and not exposed to elements such as indoor building timbers; to a maximum decay risk of 5, where wood is completely immersed or permanently exposed to salt water, for example marine timbers (piers etc.). Ground contact timbers (i.e. fence posts, telegraph poles) are categorised as hazard class 4, in which the wood is permanently exposed to wetting and has direct contact with the microbial reservoir in the soil. Soil is one of the most aggressive natural environments to which wood can be exposed (King *et al.*, 1981), since it contains both moisture and additional nutrients that are generally required by decay micro-organisms. Jellison *et al.* (1997), Palfreyman *et al.* (1996) and Srinivasan (1993) have all reported on the essential role of metal ions in wood decay activity by basidiomycetes, and it is an established fact that soft rot fungi require an external source of nitrogen in order to produce degradative enzymes (Mowe, 1983). Hardwoods are more prone to soft rot attack than softwoods such as spruce or pine, due to the higher nitrogen content of hardwood species. Figure 1.3 shows an example of the deterioration of beech blocks that have been exposed to soil.

The majority of micro-organisms occurring naturally in soil are not metabolically active. Viable spores of wood-colonising fungi do not usually germinate in the soil but remain dormant, a phenomenon referred to as fungistasis (Griffin *et al.*, 1975). This type of passive dormancy is thought to be due to either nutrient deprivation (Lynch, 1982) or a "fungistatic factor" and as a result, spore germination must somehow be induced before fungal attack takes place. Mowe (1983) proposed that wood volatiles may act as a trigger to initiate fungal spore germination and also to orient hyphal growth in the direction of the wood. It is not uncommon for wood-colonising fungi to be sub-cultured onto growth media containing sterile sawdust, in order to stimulate growth or sporulation of the fungus. The use of wood products for fungal culture revival described here demonstrates the validity of the above hypothesis regarding the induction of spore germination.

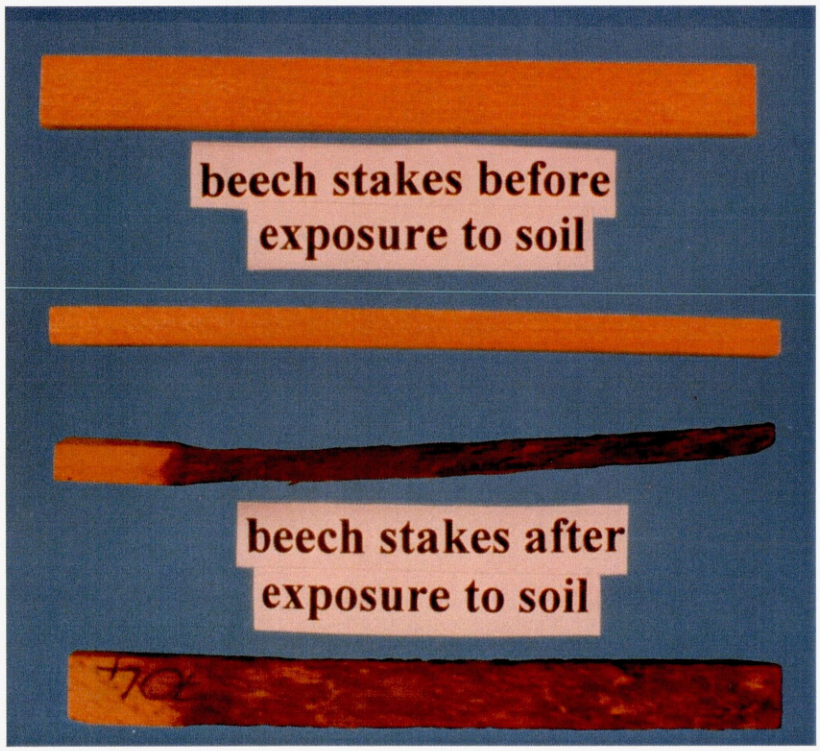


Figure 1.3: Beech blocks showing decay following exposure to soil

1.9 Colonisation of wood in-service by micro-organisms

Colonisation of ground contact timbers in-service follows a process of succession, generally starting with bacteria and primary moulds followed by non-degrading organisms such as sapstainers (Zabel and Morrell, 1992). Sapstain organisms often colonise freshly felled wood, and the blue-black discoloration observed is caused by the growth of darkly pigmented hyphae through the cells within the sapwood. While most early colonisers of wood do not cause substantial degradation, some are capable of causing physical change which can in turn influence the subsequent colonisation of decay fungi. For example, Levy (1975) wrote that bacteria have been shown to increase permeability of softwoods by the partial or total destruction of pit membranes. This effect on permeability is thought to be through the release and action of pectinolytic enzymes (Sharma and Kumar, 1979). Johnson and Gjovik (1970) reported on the effect of *Trichoderma viride* on the permeability of pine, and this effect has been exploited by researchers to improve preservative uptake in softwoods (Rosner *et al.*, 1998; Tucker *et al.*, 1998). Opened pit membranes in ground contact timbers can result in less anaerobic conditions and possibly open pathways for fungi appearing later in the succession pattern.

Primary moulds do not appear to degrade cell walls and are thought to be dependent on non-structural sugars and other simple carbohydrates present in the wood rays. Sapstain organisms tend to colonise the ray parenchyma cells of the sapwood, utilising cell contents and stored food reserves as a nutrient source (Abraham *et al.*, 1993; Breuil, 1998).

After primary colonisers such as moulds and sapstain organisms have become established, soft rot and basidiomycete wood decay fungi will colonise and begin to degrade wood. As significant biodeterioration takes place and wood cell components

are utilised by decay fungi, secondary moulds colonise the wood and become established (Mowe, 1983).

1.10 Wood decay fungi

A number of different terms have been used to describe and categorise decay since the recognition that fungi cause degradation of wood in natural and man-made environments (Eaton & Hale, 1993). Early terms include brown rot and white rot and these are still in widespread use today, although building surveyors will often define fungal decay as "dry rot" or "wet rot". In ground contact timbers however, the different decay types are more commonly referred to as brown rot, white rot and soft rot.

1.10.1 Basidiomycete decay fungi

"Dry rot" is a major source of structural timber failure in the UK and is caused by *Serpula lacrymans*, one of the most commonly known basidiomycetes. The name "dry rot" was attributed to the type of decay associated with this organism due to the appearance of dry-rotted timber rather than the conditions under which it occurs. "Wet rot" is the term used to describe decay of wooden structures within buildings or in external joinery (Eaton and Hale, 1993), which in the UK is often caused by the brown rot fungus *Coniophora puteana*. The terms brown rot and white rot are used to describe the two types of decay caused by basidiomycete fungi, based on the colour of the decayed wood. Timber which has been decayed by brown rot fungi is, as the name suggests, brown in appearance and displays cross-cracking in advanced stages of decay while white-rotted wood is bleached in colour and may have a more fibrous appearance (Eaton and Hale, 1993). The discoloration of basidiomycete decayed wood is a result of the different chemical processes involved in degradation of the wood cell wall components. Brown rot fungi are cellulolytic fungi, meaning that they target the wood

cell wall carbohydrate cellulose leaving mostly modified lignin, whereas white rot fungi possess the ability to degrade each of the three major cell wall polymers - lignin, cellulose and hemicellulose. One example of a white rot fungus is *Trametes versicolor*, often found on wounded or dead standing trees and non-ground contact timbers. White rot fungi are more commonly associated with natural decomposition of forest materials than with in-service damage, the majority of which is attributed to brown rot fungi (Eaton and Hale, 1993). Hardwoods are more susceptible to white rot decay than softwoods (Nilsson and Daniel, 1987), and as construction wood in the UK is mainly made from softwoods, white rot fungi less commonly cause decay within buildings (Schmidt and Kerner-Gang, 1986).

1.10.2 Soft rot decay fungi

Soft rot decay of wood is typically caused by members of the Ascomycotina and Dueteromycotina classes of fungi. The term soft rot was first applied by Savory (1954) to describe the surface softening of wood attacked by cellulolytic fungi. This decay occurs principally under conditions where the growth and activities of basidiomycete fungi are retarded or inhibited, *e.g.* by high moisture contents or by preservatives (Eaton & Hale, 1993) and is of particular importance when timber is used in ground contact situations. Initial colonisation of wood by soft rot fungi is principally via the rays, and also via the vessels in hardwoods.

Soft rot fungi are considered to be more tolerant of high levels of toxic chemicals than basidiomycetes (Bravery, 1975) and will often invade preservative treated timber that has been rendered resistant to basidiomycete decay (Daniel and Nilsson, 1998). They are regarded as a major cause of in-service failure of CCA-treated wood in soil (Mowe, 1983) and it appears that in such situations, where the ecological

succession pattern is altered to exclude basidiomycete decay fungi (Clubbe and Levy, 1982), soft rot fungi predominate.

Soft rot attack has been described as a series of consecutive stages, and these are listed below: -

1 - colonisation of the wood substrate

2 - hyphal penetration of the fibre walls

3 - formation of T-branches or L-branches (this is the way in which the cavity-forming hyphae orient themselves along the cellulose microfibrils of the S_2 layer)

4 - formation of cavities around the fungal hyphae

Soft rot fungi attack the cellulose-rich S_2 layer of the wood cell wall, forming longitudinal, diamond-shaped cavities (Type 1) or eroding the wood cell wall to produce erosion troughs (Type 2) (Nilsson, 1988; Daniel and Nilsson, 1998). Figure 1.4 shows an example of a soft rot cavity within a wood fibre observed using polarised light microscopy. The alignment of the chains of cavities within cell walls directly follows the helical orientation of the cellulose microfibrils. In the more advanced stages of attack when much of the S_2 has been destroyed, the cavities may become so wide and abundant that they coalesce around the wall and it becomes difficult to differentiate discrete cavities. Cavities may also develop in the S_1 layer where they follow a steeper helix along the microfibrils of this layer (Eaton and Hale, 1993).

The consequence of these cavities in the wood cell wall is that timber becomes softened or sponge-like at the wood-soil interface. As the decay develops and surface erosion occurs, the variation in density across growth rings becomes more pronounced, resulting in the corrugated appearance of severely soft-rotted wood (Eaton and Hale, 1993).

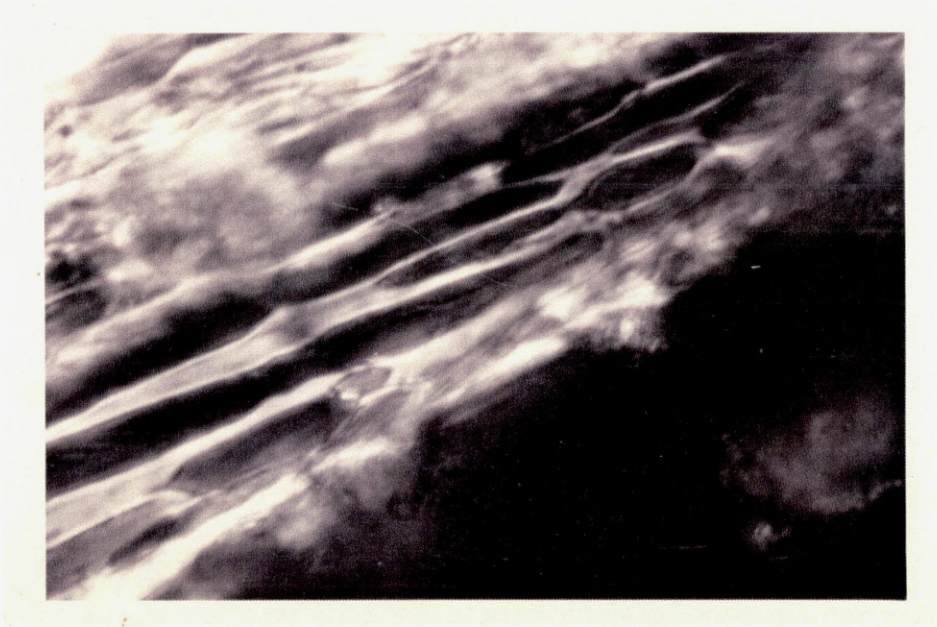


Figure 1.4: Characteristic diamond-shaped decay cavity caused by soft rot fungi

1.11 Detection of decay

The simplest methods for detecting decay in ground contact timber are subjective assessments of the condition of the wood. Sound waves can reveal the presence or absence of decay if wood is struck by a hammer - sound wood will give a sharp ring and decayed wood will sound a dull thud. If a penknife or other sharp instrument is pushed into the surface of the wood and twisted, the resulting splinter can determine whether the wood is sound or not. In decayed wood the splinter will break prematurely whereas a splinter lifted from undecayed wood will be long and difficult to detach from the wood surface.

A more quantitative assessment of surface softening can be obtained using a pilodyn (Cobra (Wood Treatment) Ltd., Brighton, UK. (Hainey, 1992)). This is a hand-held instrument containing a spring-loaded pin, set at a pre-determined pressure. The pin is fired into the wood and the depth of penetration will indicate both the presence and extent of decay. Sound wood has a higher resistance to impact than decayed wood and the pin will be unable to penetrate to any significant depth. There are, however, a

number of factors that can influence resistance to impact including the density and moisture content of the wood being tested (Hainey, 1992).

Another method to determine whether decay is present in wood is to measure the resistance to pressure, for example using a decay detection drill. A hand-drill is used to bore holes into a piece of timber; decayed wood will have decreased resistance, which is detected by a computer and areas of internal weakness can then be mapped (SIBERT Technology, *circa* 1995).

More recently developed instruments to detect the presence of decayed wood use sonic and ultrasonic soundwaves. This acoustic resonance technology can now be used to detect decay without further damaging the timber (Eaton and Hale, 1993), an important factor when dealing with decay in structural timbers, especially where historic buildings are involved. The accuracy of such methods can however be affected by timber type and structural characteristics. For example, acoustic resonance technology works well on Douglas fir and Western red cedar, but is affected by the high incidence of ring shakes in wood types such as Southern pine.

Decay can be detected using electrical resistance, where decayed wood will register reduced electrical resistance in comparison with sound wood (Eaton and Hale, 1993), however moisture content will have a significant effect on the outcome of such test methods. There has also been investigation into the detection of fungi by the analysis of volatile organic compounds (VOC's) released by dry rot fungi (Bjurman and Kristensson, 1992) and development of an "electronic nose", using sensors designed to detect volatile emissions from decay fungi (Nilsson, 1998).

1.12 Methods for fungal identification

Once the presence of decay has been established, the organisms responsible for the biodeterioration can be identified. The standard method of fungal identification is through the use of selective media systems followed by morphological comparisons with known isolates or published keys; however this is sometimes dependent on the accuracy of previously recorded descriptions of culture appearance, growth and behaviour. For an accurate identification using morphological characteristics, the organism must be grown on media that allows normal growth, and that permits the formation of fruiting bodies or other reproductive structures. For example, the addition of benomyl and streptomycin in malt agar plates will provide a medium which will inhibit mould and bacterial growth but will allow the growth of basidiomycete decay fungi (Clubbe and Levy, 1977) which can then be compared with laboratory stock cultures and taxonomic keys such as Stalpers (1978) for basidiomycetes, and Garrett (1981) or Domsch *et al.*, (1980) for soil fungi.

The development of biochemical and molecular techniques has also been applied to fungal identification. Methods such as the enzyme linked immuno-sorbent assay (ELISA) (Palfreyman *et al.*, 1991) and the polymerase chain reaction (PCR) (Edel, 1998) can be used to identify and quantify specific fungi, and as such can be used in conjunction with, or in place of, traditional methods of fungal identification.

1.13 Chemical preservation

The current traditional manner of preparing timber for use in ground contact situations in the UK is to apply toxic chemicals such as creosote or copper-chrome-arsenic (CCA) formulations using vacuum pressure impregnation. The efficacy of creosote and CCA treatment is well documented, and the methods used for chemical impregnation are tried and tested (Greaves, 1987). However, some chemicals

previously used as preservatives for ground contact timber have been seen to fail during their expected service period (Butcher, 1972; Schmidt and Kerner-Gang, 1986) and in addition the safety of such chemical preservatives is now under ever increasing scrutiny (Barnes, 1993; Murphy and Dickinson, 1997).

Chemical wood preservatives can be divided into three categories, oil-based, water-borne and organic solvent-based chemical preservatives. The preservative oils are derived from coal tar, petroleum or wood tar. Creosote, an oil-based preservative derived from coal tar, has been a dominant wood preservative since the mid-1800s (Murphy and Dickinson, 1997).

The water-borne chemicals are formulations of toxic salts such as copper-chrome-arsenic (CCA) or ammoniacal copper chromate (ACA) which become fixed in the wood structure e.g. the fixative agent in CCA is a compound of hexavalent chromium which binds to hydroxyl groups in the wood cell walls (Green, 1988). Water-borne preservatives are sub-divided into fixed (those applied using vacuum-pressure impregnation processes) and unfixed (applied by dipping or diffusion processes) formulations.

The third category of chemical preservatives contains chemicals toxic to fungi and insects, which are only soluble in organic solvents. Light organic solvent preservatives (LOSP) containing organic biocides such as tributytin oxide (TBTO) were introduced in the 1960s along with pressure treatments to combat specific problems of early decay of softwood exterior joinery which had been treated using a vacuum process (Murphy and Dickinson, 1997).

1.14 Alternatives to chemical preservation

Over the past decade, increasing concerns about the environmental impact of chemical preservatives have affected commercial wood treaters and major users of treated wood products. Pentachlorophenol (PCP), inorganic arsenics and creosote - the most commonly used wood preservatives - are now restricted use pesticides in many countries (Zabel and Morrell, 1992). There are also problems associated with the disposal of treated wood and soil contaminated as a result of chemical wood preservation. Environmental considerations such as these, along with the need for longer lasting protection and reduced costs, have led to a substantial increase in research aimed at developing safer, more environmentally acceptable methods of wood preservation.

There have been several proposed mechanisms for reducing the use of chemically preserved timber. As the use of naturally durable wood species may not be appropriate for all in-service applications, alternative strategies have been researched including the use of less toxic chemicals such as borates (Pizzi, 1998); integrated control, combining the use of chemical and biological control agents (Srinivasan, 1993; Behrendt *et al.,* 1995); sheathing, where a polyethylene lining is placed between the timber and the soil to act as a physical barrier (Baecker, 1993; Behr *et al.,* 1997); physical modification of wood, through ultra-high temperature heat treatments (such as pyrolysis and retification) which increases the physical durability of the wood structure (Dirol and Guyonnet, 1993); and chemical modification of wood, such as ion precipitation, aimed at inhibiting the biochemical activity of wood decay fungi (Green III *et al.*, 1997). This project examines the use of biological control (*i.e.* the use of micro-organisms or their products to inhibit colonisation and decay by wood-degrading fungi (Freitag *et al.*, 1991)) as a proposed alternative to chemical treatment.

1.15 Biological control

An early definition of biological control is that given by Smith (1948):

"The suppression of a pest by means of the introduction, propagation and dissemination of the predators, parasites and diseases which attack it".

However, a more appropriate definition when discussing the use of biological control in a variety of systems is:

"The reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists." (Baker and Cook, 1974).

Suppression of wild rabbit populations through the introduction of the myxamatosis virus is a classic example of the use of biological control strategies. Biological control is now well-established as a means of reducing the incidence of a variety of pests in agricultural systems. Nematodes, weeds, insects and microbial plant pathogens have all been successfully controlled using antagonistic organisms (Bruce, 1998; Schoeman *et al.*, 1999; Ricard, 2002) and biological control agents such as *Trichoderma* spp. have been used effectively in agriculture for several years (Nelson *et al.,* 1987; Rodgers, 1989; Hjeljord and Tronsmo, 1996; Elad and Freeman, 2002).

Although biological control of wood decay has been a major area of research for a number of years, there is one major specific difference between biological control strategies applied to agriculture and those applied to forest products. In general, agricultural crops only require protection from disease during their growing season, in most cases less than a year (Freitag *et al.*, 1991). A successful strategy for the biological control of wood decay would require complete protection from a wide

range of micro-organisms with varying physiological capabilities for anything up to 40 years' service life (Bruce, 1998). There are three major applications of biological control with regard to wood decay:

1. preventing fungal stain in freshly sawn lumber,
2. preventing colonisation of stored wood or wood products by decay fungi,
3. eliminating incipient fungal colonisation in various types of in-service timber and timber products.

The complexity of the requirements for adequate biocontrol strategies to prevent wood decay under a variety of circumstances makes bioprotection difficult to apply in wood preservation (Freitag *et al.*, 1991), particularly with regard to ground contact timbers. A range of potential biocontrol agents including bacterial, yeast and fungal isolates have been tested to assess their antagonistic properties against wood-degrading organisms. For example, *Pseudomonas* spp. and *Bacillus* strains have been reported to affect both sapstain and wood decay fungi in laboratory tests (Benko, 1988; Schoeman *et al.*, 1999); the yeast *Debaryomyces hansensii* has been tested for its ability to control molding and staining fungi in pine lumber (Payne and Bruce, 2001); and a considerable amount of research has taken place into the assessment of fungal biocontrol agents (Bruce, 1998; Schoeman *et al.*, 1999).

Isolates selected using laboratory-based screening systems, however, have often been shown to be less successful under field conditions. Laboratory screening studies have indicated that selected *Trichoderma viride* isolates are effective in protecting Scots pine and Sitka spruce against decay caused by basidiomycete fungi (Tucker *et al.,* 1996), although little or no research into the biocontrol of wood decay fungi has targeted soft rot organisms.

Research into the suitability of potential biocontrol agents must also consider aspects such as method of control, nutritional status of the substrate and surrounding environment, and application method. The delivery formulation and timing of the application of a potential biocontrol agent are as important as the selection of an appropriate antagonist. Novel application methods have been developed with regard to biocontrol agents, such as BINAB pellets and chainsaw oil (Bruce, 1983; Schoeman *et al.*, 1994; Bruce, 1998), however delivery methods must also be appropriate to the end use of the treated timber. Incomplete establishment of biocontrol agents has been reported previously as a potential factor in the failure of biological control strategies.

Many of the reported field trials in biocontrol of wood decay such as those carried out by Bruce, for example, have dealt with remedial treatment of preservative-treated in-service timbers e.g. electricity distribution poles and have targeted a narrow pest range (Bruce and King, 1986a, b; Bruce *et al.,* 1990). There have been relatively few field trials investigating the use of biocontrol agents such as *Trichoderma* spp. to protect untreated timber and the majority of those reported have been concerned with immediate post-harvest deterioration of timber, such as Schoeman *et al.* (1994). That study investigated the application of *Trichoderma* spores in chainsaw oil as a means of protecting freshly harvested wood against sapstain and basidiomycete decay organisms. The assessment of the efficacy of a selected *Trichoderma* isolate as a means of protecting wood in ground contact against a wide range of target organisms, as described in this thesis, will provide a more comprehensive evaluation of the biocontrol potential of this fungus and its suitability as a biocontrol agent for wood used in Hazard Class 4 situations.

1.16 *Trichoderma* spp. as potential biological control agents

Trichoderma spp. are a group of soil-inhabiting, non-degrading wood-colonising fungi that possess a number of specific characteristics which make them highly suitable as potential biocontrol agents (Schoeman *et al.*, 1999). They are fast-growing, tolerant to a range of chemical preservatives and have been shown to possess a wide range of antagonistic properties against a variety of agricultural and forest products pests (Bruce, 1998). Early screening studies into the potential application of biological control to forest products targeted *Heterobasidion annosum*, a major cause of root rot in conifers. Mycoparasitism was observed in interactions between *H. annosum* and several *Trichoderma* isolates taken from the same wood samples (Ricard, 1970). *Trichoderma* has since been investigated as a means of controlling root and foliar pathogens, although laboratory screening results and field performance have not always been consistent (Schoeman *et al.*, 1999). *Trichoderma* is currently the most extensively researched biocontrol fungus in the field of forest products protection, and has been shown on a number of occasions to provide a protective effect against certain wood decay fungi (Hulme and Shields, 1970; Highley & Ricard, 1988; Bruce and Highley, 1991; Bruce, 1998). One particularly outstanding feature of naturally-occurring *Trichoderma* spp. used for biocontrol purposes in agriculture is the consistency of their safety features such as lack of toxicity to humans and animals. For instance *Trichoderma* preparations used to suppress *Botrytis cinerea* on soft fruit can be applied up to harvest time (Ricard, 2002).

The mechanisms that are generally regarded as the means by which the organism *Trichoderma* protects wood from basidiomycete decay are outlined below. Mechanisms may be utilised individually or in combination with one another, further increasing the host range and activity of *Trichoderma* spp. as biocontrol agents.

(i) Competition for nutrients is a passive mechanism in that *Trichoderma* spp. are nutritionally non-exacting and fast growing, enabling them to rapidly colonise untreated wood and exhaust the readily available nutrients, thus retarding the growth of other potential colonisers (Hulme and Shields, 1970; Hulme and Shields, 1972).

(ii) Mycoparasitism involves the recognition of the target fungus by *Trichoderma*, which then attaches by coiling around the opposing hyphae or producing "hook-like" structures (Elad *et al.,* 1982). Extracellular lytic enzymes, *e.g.* chitinase, will then lyse and degrade the cell wall structure of the invading fungus (Sivan and Chet, 1989). The breakdown products of the cell wall degradation are then utilised by *Trichoderma.*

(iii) Antibiotic production can be through the production of either soluble antibiotics (Dennis and Webster, 1971a; Bruce and Highley, 1991) or volatile organic compounds (VOC's) (Dennis and Webster, 1971b; Bruce, Austin and King, 1984; Bruce *et al.*, 1996; Wheatley *et al.*, 1997). Both forms of metabolite are believed to have fungicidal or fungistatic properties (Srinivasan *et al.*, 1992a, b; Palfreyman *et al.*, 1996).

Siderophores are iron-chelating compounds that work by sequestering available iron ions vital to many micro-organisms' enzymic systems. Srinivasan (1993) studied the effect of these compounds on the inhibition of basidiomycete fungi in culture. *Trichoderma* produce siderophores which can bind the iron reported to be required by decay fungi for wood degrading activities (Jellison *et al.*, 1990). Research into the role of siderophores as a mode of antagonism employed by *Trichoderma* has implicated this as a mechanism of action against wood decay fungi (Srinivasan, 1993).

1.17 Aims of the project

Timber used in ground contact situations is exposed to attack from a wide range of biodeteriogens. At present, chemical preservatives are used to protect wood from biodegradation and thus prolong its service life. However, chemical wood preservative formulations such as creosote or CCA are generally toxic, and as a result of increasing legislation aimed at environmental protection such as Life Cycle Assessments (Murphy and Dickinson, 1997) and the European Biocidal Products Directive (CEN, 2000), a requirement for less toxic alternatives to the chemical preservation of ground contact timbers has emerged. The overall objective of this project was to investigate the efficacy of biological control as a means of preventing the biodegradation of ground contact timber by wood decay fungi. The work was broken down into a number of aims:

- To design and develop a small-scale, wood-based screening system to facilitate the rapid laboratory assessment of the biocontrol potential of a large number of isolates.

- To apply the developed screening system to assess the protective and antagonistic properties of a range of *Trichoderma* isolates, in order to select a suitable biocontrol agent for use in a larger-scale test system.

- To assess the compatibility of biological control with standard industry processes, through the use of a pilot preservation plant to pressure-impregnate wood with a fungal spore suspension.

- To implement a large-scale test system to observe the biocontrol potential of the selected isolate to determine the efficacy of the isolate under conditions similar to the proposed end use of chemically preserved timber.

- To evaluate the use of an accelerated decay facility to observe the selected isolate in conditions more conducive to extensive decay, and comparing fungal cellar results with those from the field trial.

- To develop a detection system using molecular biology techniques such as the polymerase chain reaction (PCR) and image analysis to assess the distribution of a *Trichoderma viride* isolate in wood treated with a spore suspension.

Chapter 2

Preliminary Screening

2.1 Introduction

In order to select a suitably antagonistic organism for use as a biocontrol agent, preliminary screening may be carried out in the laboratory to give an indication of the biocontrol potential of proposed isolates. Species such as *Trichoderma*, *Gliocladium* and *Scytalidium* have all been shown to be potential biocontrol agents using laboratory screening tests (Bruce and King, 1986 a; Murmanis *et al.*, 1988; Highley and Ricard, 1988). There are a number of commonly used methods for screening potential biocontrol agents (Seifert *et al.*, 1988; Schoeman & Dickinson, 1993; Archer *et al.,* 1993), and each of these methods has its own advantages and disadvantages.

Agar plate interaction studies have been used to select a number of biocontrol strains of *Trichoderma* for the protection of ground contact timbers (Tucker *et al.*, 1997). Agar plate studies are a fast, simple and inexpensive method of detecting antagonistic activity of potential biocontrol agents, however the use of selective media may be needed to verify the outcome of the interaction in the event of apparent death of the target organism. In addition to this, the composition of the media may not accurately represent the nutritional or physical characteristics of wood (Tucker *et al.*, 1997). New media have been developed which are more representative of the nutrient conditions of wood, for instance, the low nutrient medium (LNM) developed by Hutterman and Volger (1973) has a carbon to nitrogen ratio similar to that of wood (Tucker *et al.*, 1997). Other low nutrient media with more specific modifications may also be used, for example, low-nutrient pine medium (Tucker and Bruce, 1995) contains the major representative amino acids in Scots pine sapwood (Nayagam, 1987).

Other systems such as agar-block tests and soil-block tests are a more comprehensive method of determining the protective effect of an organism, but require too long an incubation period for a preliminary screening system. Modifications of European standards such as EN 113, "Wood preservatives. Determination of the toxic values against wood destroying Basidiomycetes cultured on an agar medium" (CEN, 1996) can subsequently be used to test potential biocontrol agents that have been identified using a preliminary screening system (Tucker *et al.,* 1996).

Another method for assessing the antagonistic activity of a potential biocontrol isolate is to use wood-soil interaction studies or soil burial methods. In these types of tests, wood blocks can be treated with either a novel chemical preservative or a biocontrol agent and tested along with reference chemical preservative controls and untreated controls. European standard methods for chemical preservatives could be modified for this purpose, however these are often designed to assess the rate of decay in larger wood samples (as in test II of the European Standard ENV 807, "Wood preservatives - Determination of the toxic effectiveness against soft rotting micro-fungi and other soil inhabiting micro-organisms" (CEN 2001), sample dimensions are 100 mm x 10 mm x 5 mm). As a result, soil burial tests are usually perceived as time-consuming and expensive and so not suitable for a primary screening method for large numbers of potential biocontrol agents. Soil burial tests using non-sterile soil are, however, likely to be particularly suitable for screening biocontrol agents to protect wood in ground contact from soft rot organisms. Soil burial testing ensures that test samples are exposed to the wide range of organisms present in soil, and also provides a suitable nutrient status to promote optimum decay activity (Gray, 1986).

While interaction studies carried out on agar may be reproducible and provide results rapidly, they do not always correlate well with results when repeated using wood (Freitag and Morrell, 1990). In addition to this, no single selective media has been successfully developed for the isolation of soft rot fungi, which makes confirming the outcome of interactions measured by the death of the target fungus extremely difficult. Therefore a soil-burial system needed to be developed as a rapid, reproducible, comprehensive screening method for determining the efficacy of potential biocontrol agents against soft rot fungi.

As hardwoods have a higher nitrogen content than softwoods and therefore are decayed more rapidly in situations such as soil burial, the hardwood beech was used to establish the screening system, designed as a rapid indicator of biocontrol capacity. Although mould fungi such as *Trichoderma* spp. have been a major focus of the investigations into wood decay biocontrol, micro-organisms from other genera have also been researched and tested as bioprotectants. For example, bacterial strains have been used to reduce sapstain discoloration of wood and wood products (Benko, 1989; Breiul, 1993).

The screening method described in this chapter has been developed in response to the need to produce a system which maximises the advantages of existing methods such as agar interactions and larger scale wood-based studies, and minimises the disadvantages. Using smaller wood block dimensions and a smaller test environment allows rapid screening of a number of potential isolates on wood in the first instance, while eliminating the need for agar based studies when screening for potential antagonists against soft rot fungi in ground contact timber.

2.2 Materials and methods

Culture maintenance and media preparation were carried out as described in Appendix A: Culture Methods and Isolation. Chemical suppliers are detailed in Appendix B: Chemicals, Reagents and Suppliers.

2.2.1 Development of screening system

Based on its performance in previous wood block studies, *Trichoderma viride* (Persoon ex S. F. Gray) isolate T60 was selected for use in the development of a small-scale, wood-based screening system. This isolate has been shown to be extremely effective in protecting wood blocks from attack by specific basidiomycetes (Tucker *et al.,* 1997) but had not been tested against soft rot fungi or non-sterile soil. A stock culture of T60 was grown on a 3% malt extract agar plate. A spore suspension was prepared by pouring 20 ml of sterile distilled water onto the plate surface, agitating the surface of the culture with a sterile inoculating loop and decanting the water from the plate. The concentration of T60 spores was calculated using a haemocytometer and the prepared T60 suspension was then diluted to give a final spore concentration of 6×10^6 spores per ml.

A total of 78 beech (*Fagus sylvatica*) blocks (fully oriented, 30 mm x 10 mm x 3 mm) were numbered and oven-dried at 105°C for 4 hours. These were cooled in a dessicator and weighed to establish the original dry weight of each block. The wood blocks were sterilised by autoclaving at 121°C for 20 minutes.

Forty two of the sterile wood blocks were dipped into the spore suspension for 5 seconds. These blocks were placed into 28ml bottles containing a flat surface of sterile soil set up at 95% of its pre-determined water-holding capacity (WHC; see Appendix C) to ensure that sufficient moisture was maintained. Figure 2.1 shows the set-up for this

experiment. The soil used was collected from soil beds present in a fungal cellar (see Chapter 4, section 4.2.1) and was sterilised by autoclaving at 121°C for 60 minutes.

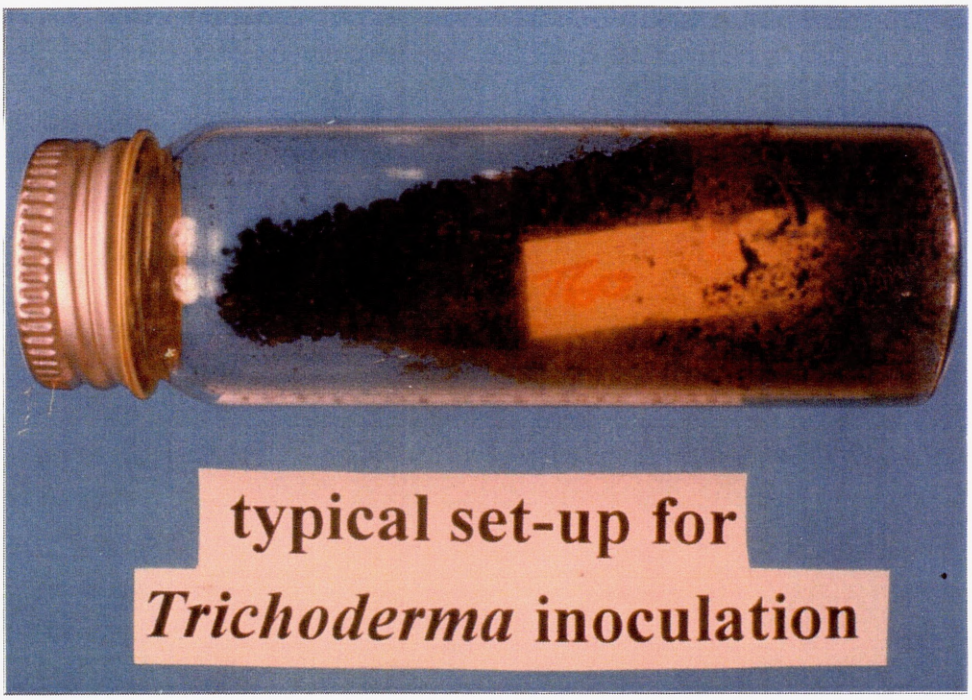


Figure 2.1: Incubation of wood blocks treated with a *Trichoderma* isolate

After two weeks incubation at 25°C to allow *Trichoderma* colonisation, the blocks were removed from the incubation bottles and buried in bottles containing either non-sterile soil (again set up at 95% WHC), or sterile soil inoculated 2 weeks previously by placing one 10 mm core of the soft rot fungus *Chaetomium globosum* Kunze (ATCC 6205) into each bottle. Untreated control blocks were also placed in bottles containing either non-sterile soil or *C. globosum*-infected soil. The remaining 6 blocks inoculated with T60 were removed, weighed and oven dried, then re-weighed to determine whether any weight loss had occurred as a result of *Trichoderma* infection. The bottles were incubated at 27°C and 75% RH for up to 7 weeks and samples (6 replicates for each category) were removed after 3, 5 and 7 weeks.

Calculation of weight loss

Six replicate blocks of each treatment were uplifted at 3, 5 and 7 weeks. The blocks were immediately weighed and the final wet weight of each block was recorded. The blocks were then oven-dried for 4 hours at 105°C and re-weighed. Weight losses were calculated using the following equation:-

$$\frac{\text{original dry weight - final dry weight}}{\text{original dry weight}} \times 100$$

Moisture content determination

Moisture content was also calculated for each block, using the following equation:-

$$\frac{\text{final wet weight - final dry weight}}{\text{final dry weight}} \times 100$$

2.2.2 Screening of potential *Trichoderma* spp. biocontrol isolates

Once the suitability of the test method had been established using T60 (see Tables 2.1, 2.2), a further 10 *Trichoderma* isolates were screened using non-sterile soil. As before, the blocks were exposed to *Trichoderma* isolates in sterile soil for 2 weeks before being incubated in non-sterile soil for 3 weeks. The isolates used were *T. viride* (Persoon ex S. F. Gray) isolates T14, T28, T40, T53, T70, T100, T110 and IMI 49791 and *T. pseudokoningii* (Rifai) isolates T51 and T64. With the exception of isolate IMI

49791, all *Trichoderma* isolates used were supplied by E. Nelson (USDA Forest Serv., Pacific Northwest Res. Sta., Oregon, USA). In some non-sporulating cultures, wood blocks were inoculated by placing a single 10mm core of *Trichoderma* from freshly produced cultures onto each sample block (see Table 2.3).

2.2.3 Bacterial screening

The protective properties of certain strains of bacteria have been reported by a number of authors (Benko, 1988; Croan, 1996). A bacterial isolate found inhibiting mould fungi on a laboratory petri dish was sub-cultured onto 3% malt extract agar (MEA), and tentatively identified as a strain of *Pseudomonas* spp. The antagonistic behaviour of the bacterial culture was assessed against selected soft rot fungi. Interactions were carried out on both 3% MEA and a low nutrient media (LNM; see Appendix A) based on Huttermann and Volger (1973), using a single *Pseudomonas* bacterial isolate (Pb1) against *T. pseudokoningii* (T64) and five soft rot fungi (*Chaetomium globosum*, *Humicola grisea* Traen (MG 28), *Trichurus spiralis* Hasselbr. (MG 31), *Petriella setifera* (Alf. Schmidt) Curzi (MG 50), and *Phialophora mutabilis* (van Beyma) W. Gams and McGinnis (S 24-E). Five replicates of each interaction were prepared with the bacterium being applied on one half of the plate as a smear using a sterile inoculating loop while the fungi were inoculated as a single core (10 mm) at the opposite side of the plate. The plates were incubated at 25^{o}C and visually assessed every 2-3 days. Following the observed inhibition of all five soft rot fungi by *Pseudomonas* in the interaction study, two wood-based screening systems were then employed to assess the biocontrol potential of the bacterium.

2.2.3.1 Soil-burial of beech wafers

Liquid cultures of the *Pseudomonas* isolate were prepared using yeast peptone dextrose (YPD) broth as described by Benko (1989). Fifty four sterile, oven-dried and weighed beech blocks (see 2.2.1 for dimensions and orientation) were treated with either a bacterial broth suspension, prepared by drawing an inoculating loop over the surface of a bacterial culture then 'washing' the loop in a flask containing 50 ml of sterile YPD broth and incubating the flask at 30°C for 3 days; or the supernatant of an identical bacterial suspension, prepared by centrifuging a culture at 4000 rpm for 15 mins. Blocks were dipped into the respective solutions for 10 seconds, while untreated control blocks were dipped into sterile water to provide a moisture level similar to the treated blocks. The wood blocks were then buried in non-sterile soil at 100% WHC. A higher WHC was used since bacteria require significantly more moisture than fungi for colonisation of wood (King, 1981). The bottles were then incubated at 26°C, 80% RH for 3, 5 and 7 weeks, at which times 6 replicate blocks for each treatment and control regime were uplifted. Moisture contents and weight losses were calculated as described previously.

2.2.3.2 Pure culture screening using beech wafers

Fifty four plates of each of the 5 soft rot test fungi were set up using 5% malt extract / 2% agar and were incubated at 25°C, 75% RH for 2 weeks. Two hundred and eighty eight beech blocks (of the same dimensions and orientation as in section 2.2.1) were prepared as above (90 blocks for each treatment + 18 controls to determine any weight losses caused by the bacteria), i.e. dipped in *Pseudomonas* culture broth; dipped in *Pseudomonas* culture supernatant; untreated controls dipped in sterile water. Eighteen replicates of each treatment were placed onto the pre-

inoculated agar plates. The plates were incubated at 25^{o}C, 75% RH for the run of the experiment and 6 replicate blocks for each treatment and test fungus were uplifted at 5, 7 and 9 weeks. Moisture contents and weight losses were calculated as before. An additional bacterial broth culture control was also set up in which blocks were treated with the bacterial suspension in the same way as before, and placed on un-inoculated agar plates. This was to observe whether the bacterial culture may have had any effect on the weight of the wood blocks.

2.3 Results

2.3.1 Development of a screening system

The screening of isolate T60 using the soil burial system allowed the determination of the overall protective effect of the isolate as well as confirming that the use of non-sterile soil in this miniaturised test system provides an extremely suitable medium for a preliminary screening system. The results presented in Table 2.1 are the mean weight loss and moisture content values for 6 replicate blocks uplifted from each treatment group. The weight loss values show that, after 3 weeks, there was significant decay of the controls in both test systems. The moisture contents of the test blocks were all well above fibre saturation point indicating that test conditions were suitable for the onset of decay.

Although there was no reduction in weight loss in T60-treated wood buried in soft rot-infected soil when compared with the untreated control, T60-treated wood exposed to non-sterile soil showed significantly less decay after 3 weeks compared with the corresponding untreated control. Statistical comparisons were carried out using one-way ANOVA tests. The reduction in decay is no longer apparent in results recorded after 5 weeks or 7 weeks soil burial, indicating that although there may initially have been a slight protective effect, no overall long-term biocontrol was achieved.

Table 2.2 shows that the weight loss caused by colonisation of beech blocks by T60 was minimal (less than 1%), and that the blocks were maintained at a moisture content at or above fibre saturation point. It is clear from these results that there was no physical degradation of the wood block by the potential biocontrol organism and that the weight loss was more likely to have been caused by the utilisation of readily available soluble sugars in the wood by *Trichoderma*.

Time (weeks)	non-sterile soil system		*C. globosum* system	
	pre-treated	untreated	pre-treated	untreated
3	7.06 (1.14)*	10.81 (2.24)	13.18 (2.48)	13.71 (2.34)
5	15.06 (2.55)	18.32 (4.97)	25.02 (3.88)	23.44 (8.10)
7	20.87 (3.53)	23.97 (3.31)	23.08 (4.76)	23.68 (4.44)

Table 2.1(a): Weight loss (%) recorded in beech blocks over screening period.

Time (weeks)	non-sterile soil system		*C. globosum* system	
	pre-treated	untreated	pre-treated	untreated
3	54.99 (4.66)	60.55 (7.79)	67.29 (9.15)	64.79 (9.49)
5	63.82 (7.39)	65.95 (16.19)	87.27 (12.88)	88.25 (20.67)
7	75.28 (10.76)	66.30 (6.90)	76.93 (16.32)	79.06 (13.04)

Table 2.1(b): Moisture contents (%) of beech blocks over screening period.

Table 2.1: Weight loss and moisture contents recorded in *Trichoderma*-treated and control beech blocks after 3, 5 and 7 weeks exposure to soil or *C. globosum.*
Figures in parentheses represent standard errors (* denotes a value that is significantly different from the corresponding control using ANOVA).

% Weight Loss	% Moisture Content
0.69 (0.25)	35.89 (2.02)

Table 2.2: Weight loss (%) and moisture content (%) of beech blocks inoculated with *T. viride* (T60) and incubated on sterile soil for 2 weeks.

2.3.2 Screening of potential *Trichoderma* spp. biocontrol isolates

Table 2.3 presents weight loss and moisture content values for 10 *Trichoderma* isolates screened using the developed test system. Although there was a small but significant reduction in the amount of decay in blocks pre-treated with *T. viride* isolate IMI 49791, none of the other isolates screened demonstrated any biocontrol potential. As with the results recorded during development of the screening system (Table 2.1 (b)), moisture content values for each of the treatment groups show moisture levels suitable for the onset of decay to occur. The screening studies were carried out in two groups, each with an accompanying untreated control group. Comparison of the weight losses achieved in these control groups demonstrated a degree of uniformity in the system.

Isolate screened	inoculum	% weight loss	% moisture content
T. viride T28	mycelial core	19.91 (10.13)	79.06 (12.81)
T. viride T40	mycelial core	18.73 (9.19)	86.51 (12.07)
T. viride T53	mycelial core	17.78 (9.79)	81.29 (15.08)
T. viride T100	mycelial core	17.20 (4.51)	86.50 (15.49)
T. viride IMI 49791	mycelial core	13.32 (5.20)*	78.88 (12.37)
control 1		19.28 (2.88)	85.05 (4.94)

Table 2.3(a): Screening group 1

Isolate screened	inoculum	% weight loss	% moisture content
T. viride T14	mycelial core	21.85 (9.71)	59.72 (7.55)
T. pseudokoningii T51	spore solution	15.43 (6.72)	78.61 (15.22)
T. pseudokoningii T64	spore solution	15.31 (6.60)	78.99 (12.90)
T. viride T70	mycelial core	15.26 (4.00)	80.08 (7.18)
T. viride T110	mycelial core	19.44 (8.50)	49.49 (8.71)
control 2		16.18 (2.07)	81.36 (5.21)

Table 2.3(b): Screening group 2

Table 2.3: Weight loss (%) and moisture content (%) of *Trichoderma*-treated and control beech blocks after 3 weeks exposure to non-sterile soil.

Figures in parentheses represent standard errors (* denotes a value that is significantly different from the corresponding control using ANOVA).

2.3.3 Bacterial screening

Agar plate interaction studies on malt extract agar showed a moderate degree of inhibition of 3 of the soft rot organisms *P. mutabilis*, *P. setifera* and *C. globosum* by the bacterial isolate. However, minimal inhibition was observed with regard to *H. grisea*, *T. spiralis* and *T. pseudokoningii* isolate T64 (see Table 2.4; Figure 2.2 (a-f)). Although none of the soft rot fungi were killed by the bacteria in the observed interactions, one of the fungi (*C. globosum*) appeared to undergo a stress reaction, producing droplets of a gold-coloured liquid at the active edge of edge of the culture and producing thick hyphal strands from the growing edge. Other reactions observed included growth of mycelia onto the exposed plastic surfaces at the sides and top of the petri dish. There was little spread of the bacterial isolate but in many of the interactions, the growing edges of the fungi were distorted in relation to normal uniformly radial growth (see Figure 2.2 (a-f)). The bacterial isolate did not grow successfully on the LNM plates, and no results were recorded for interactions carried out on this media.

Test organism	Degree of inhibition
Humicola grisea (MG 28)	++
Chaetomium globosum (ATCC 6205)	++
Trichurus spiralis (MG 31)	+
Petriella setifera (MG 50	+
Phialophora mutabilis (S 24-E)	+
Trichoderma pseudokoningii (T64)	++

Table 2.4: Extent of inhibition of selected mould fungi by the bacterial *Pseudomonas* isolate observed in agar plate interaction studies.

Key: + - slight degree of inhibition of target fungus
++ - moderate inhibition and distortion of growing edge
+++ - severe inhibition and/or apparent death of target fungus

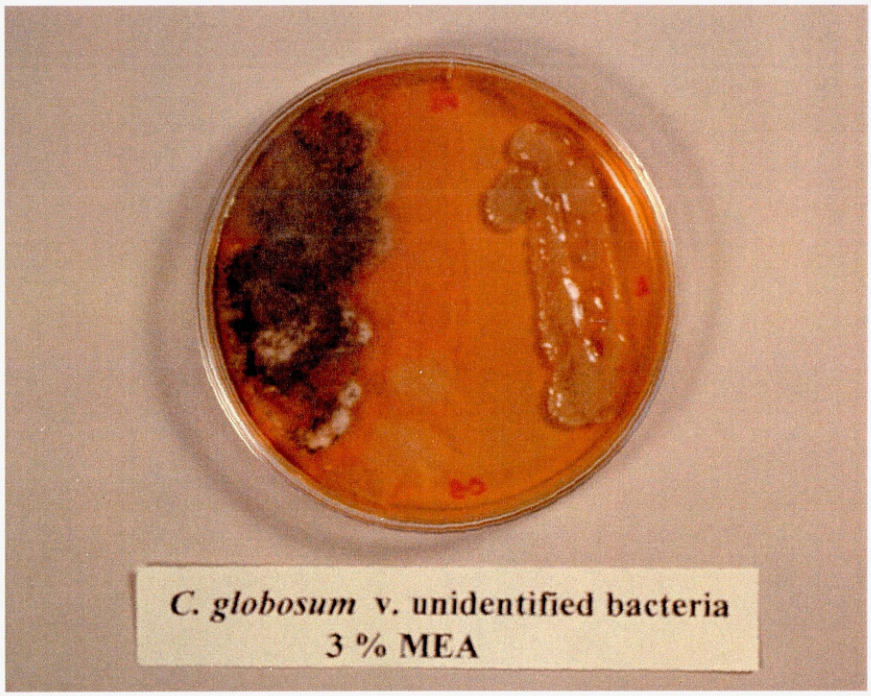


Figure 2.2 (a): *Pseudomonas* isolate vs. *C. globosum*

Figure 2.2 (b): *Pseudomonas* isolate vs. *H. grisea*

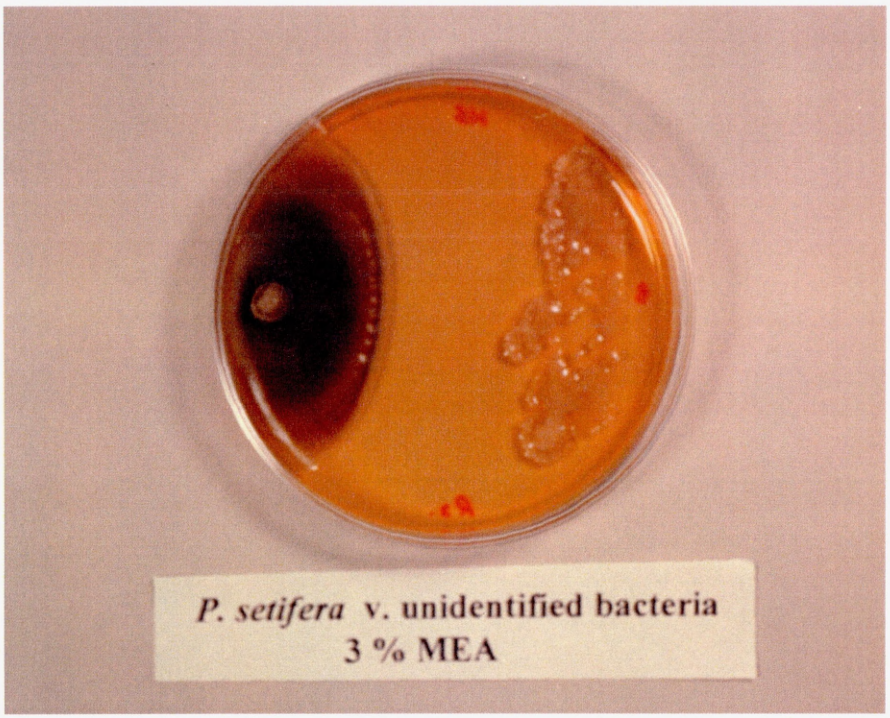


Figure 2.2 (c): *Pseudomonas* isolate vs. *P. setifera*

Figure 2.2 (d): *Pseudomonas* isolate vs. *P. mutabilis*

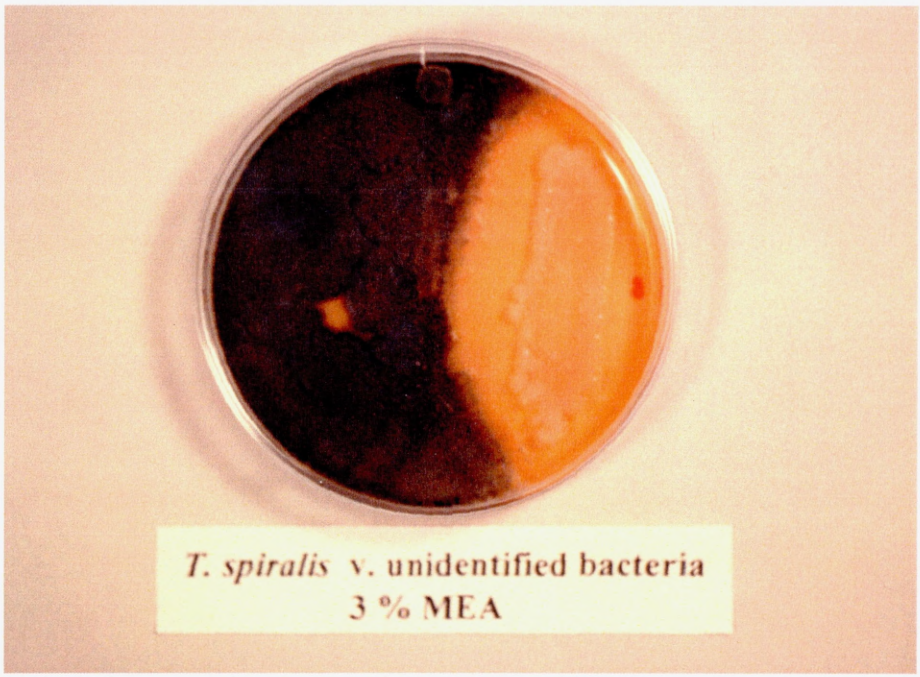


Figure 2.2 (e): *Pseudomonas* isolate vs. *T. spiralis*

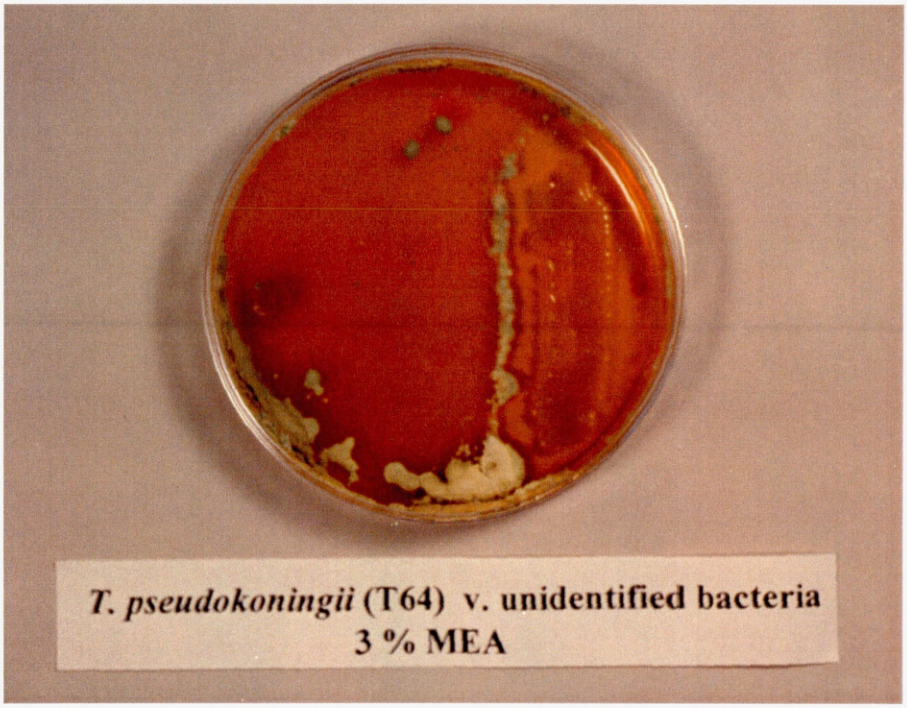


Figure 2.2 (f): *Pseudomonas* isolate vs. *T. pseudokoningii*

Figure 2.2: Interaction plates of *Pseudomonas* against selected soft rot fungi and *Trichoderma pesudokoningii* (T64)

2.3.3.1 Soil-burial of beech wafers

The results for *Pseudomonas*-treated wood buried in non-sterile soil are presented in Table 2.5 and show particularly high weight loss values, especially from the final uplift after 7 weeks exposure. The moisture contents of the blocks were also high, although this apparently had little effect on the biodegradative action of the soil organisms and simply indicates the occurrence of large amounts of decay. No significant reduction in the amount of decay was recorded in this experiment. Indeed, blocks treated with the bacterial supernatant appear to have a slightly higher degree of decay, however this was not a statistically significant increase.

	Treatment		
Time (weeks)	**broth**	**supernatant**	**control**
3	14.78 (1.30)	17.30 (1.04)	15.38 (3.74)
5	29.35 (8.04)	35.51 (6.53)	31.62 (4.26)
7	39.42 (5.39)	40.07 (6.52)	35.81 (3.46)

Table 2.5(a): Weight losses (%) recorded in beech blocks.

	Treatment		
Time (weeks)	**broth**	**supernatant**	**control**
3	90.91 (9.28)	93.65 (4.89)	99.59 (14.08)
5	125.56 (44.69)	133.80 (25.12)	123.65 (14.35)
7	137.10 (26.25)	144.77 (29.63)	125.19 (12.45)

Table 2.5(b): Moisture contents (%) measured in beech blocks.

Table 2.5: Weight loss and moisture contents of beech blocks treated with either bacterial broth or supernatant and untreated control blocks following 3, 5 and 7 weeks exposure to non-sterile soil.

Figures in parentheses represent standard errors.

2.3.3.2 Pure culture screening using beech wafers

Pure culture screening of the bacterial isolate using agar plates of the 5 selected soft rot fungi showed a high degree of variation (Table 2.6). A large proportion of the results from this experiment showed weight losses below 3%, and high moisture contents were also recorded (Table 2.7). Typically, weight losses below 3% are not considered to be significant, as this is generally attributed to the utilisation of soluble sugars by colonising organisms. The high moisture contents recorded in this experiment are not associated with major weight loss in this instance, and may account for some of the low weight loss results.

The treatment groups which displayed significant weight losses were statistically analysed using one-way ANOVA. Blocks treated with a broth culture of the *Pseudomonas* isolate and exposed to *C. globosum*, *T. spiralis* and *P. setifera* all showed significant reductions in weight loss after 5 weeks. Although treated blocks exposed to *H. grisea* did demonstrate a reduction in weight loss after 5 weeks when treated with *Pseudomonas* broth, this was not found to be statistically significant due to variability between replicate samples. Samples treated with bacterial broth and then exposed to *P. mutabilis* showed an increase in weight loss after 5 weeks as compared with the untreated controls, however this effect was not seen in the weight loss results after further exposure. Overall, the results of this experiment do not appear to follow any consistent pattern. There was no uniformity even in the amount of decay recorded in untreated blocks; for example, untreated control blocks exposed to *C. globosum* displayed around 11% weight loss after 5 weeks, yet after 7 weeks the mean weight loss was less than 2%.

The lack of consistency in the results was also apparent in the blocks used to determine the effect of the bacteria alone on wood blocks (Table 2.8). After 5 weeks,

the blocks had lost an average of 3% of their dry weight. After 7 weeks and 9 weeks, the mean weight of the blocks had increased by around 3%. This may have been due to an increase in biomass as a result of bacterial colonisation. However, as the test blocks were placed directly onto the surface of the fungal cultures, it is possible that the observed weight increases may have been caused by absorption of nutrients by the wood.

Time (weeks)	broth	supernatant	control
5	4.59 (1.85)	6.41 (1.66)	7.73 (2.71)
7	5.74 (3.02)	8.10 (3.40)	8.52 (3.94)
9	9.08 (3.55)	9.92 (4.00)	9.22 (3.33)

Table 2.6 (a): *H. grisea*

Time (weeks)	broth	supernatant	control
5	5.14 (2.60)*	11.11 (3.45)	11.26 (2.48)
7	2.73 (2.58)	6.35 (3.32)	1.45 (1.28)
9	3.08 (1.92)	3.94 (1.84)	2.90 (1.84)

Table 2.6 (b): *C. globosum*

Time (weeks)	broth	supernatant	control
5	3.80 (1.61)*	7.16 (1.51)	5.50 (0.68)
7	2.24 (1.30)	2.02 (0.82)	3.42 (0.79)
9	2.53 (0.76)*	2.20 (0.83)*	4.88 (0.83)

Table 2.6 (c): *T. spiralis*

Time (weeks)	broth	supernatant	control
5	2.45 (1.58)*	4.99 (1.44)	6.28 (1.68)
7	+0.10 (0.60)	0.43 (0.47)	0.91 (0.49)
9	+0.31 (0.74)	0.65 (0.43)	0.74 (0.45)

Table 2.6 (d): *P. setifera*

Time (weeks)	broth	supernatant	control
5	7.24 (1.41)*	1.50 (2.96)	4.19 (1.93)
7	3.32 (2.26)	0.105 (1.11)	2.53 (2.13)
9	5.97 (2.62)	3.18 (3.40)	4.16 (1.24)

Table 2.6 (e): *P. mutabilis*

Table 2.6: Weight loss (%) of beech blocks treated with either bacterial broth or supernatant and untreated control blocks following 5, 7 and 9 weeks exposure to pure cultures of selected soft rot fungi.

Figures in parentheses represent standard errors (* denotes a value that is significantly different from the corresponding control using ANOVA).

Time (weeks)	broth	supernatant	control
5	83.73 (11.32)	90.79 (9.21)	111.77 (18.91)
7	98.14 (10.75)	88.57 (11.95)	118.24 (8.18)
9	110.56 (14.85)	96.42 (12.16)	121.32 (6.08)

Table 2.7 (a): *H. grisea*

Time (weeks)	broth	supernatant	control
5	86.74 (10.11)	96.96 (12.36)	120.78 (13.82)
7	92.18 (11.94)	90.48 (11.19)	102.85 (3.81)
9	91.97 (15.99)	85.52 (5.77)	110.82 (6.24)

Table 2.7 (b): *C. globosum*

Time (weeks)	broth	supernatant	control
5	81.54 (9.25)	97.87 (13.91)	94.42 (9.00)
7	87.81 (10.34)	83.45 (6.54)	91.59 (11.59)
9	84.35 (8.49)	97.59 (10.20)	95.83 (9.57)

Table 2.7 (c): *T. spiralis*

Time (weeks)	broth	supernatant	control
5	85.41 (9.60)	109.63 (6.01)	106.20 (7.41)
7	77.02 (9.44)	97.16 (5.61)	91.86 (9.96)
9	90.32 (11.12)	99.60 (4.55)	90.87 (8.58)

Table 2.7 (d): *P. setifera*

Time (weeks)	broth	supernatant	control
5	106.34 (8.41)	99.75 (5.23)	97.59 (8.75)
7	88.60 (7.08)	99.40 (2.57)	96.96 (5.16)
9	105.55 (5.91)	99.24 (9.17)	99.70 (3.39)

Table 2.7 (e): *P. mutabilis*

Table 2.7: Moisture content (%) of beech blocks treated with either bacterial broth or supernatant and untreated control blocks following 5, 7 and 9 weeks exposure to pure cultures of selected soft rot fungi.

Figures in parentheses represent standard errors (* denotes a value that is significantly different from the corresponding control using ANOVA).

Time (weeks)	% weight loss	% moisture content
5	3.10 (1.85)	104.08 (11.55)
7	+2.13 (0.93)	93.22 (10.77)
9	+3.28 (0.46)	97.20 (8.10)

Table 2.8: Weight loss (%) and moisture content (%) of beech blocks dipped in bacterial broth and placed on agar for 2 weeks.

2.4 Discussion

Trichoderma viride isolate T60 was selected as a starting point in this experiment on the basis of its effectiveness as an antagonist of wood decay fungi. Studies carried out both on agar plates and using wood block systems demonstrated the biocontrol potential of this isolate (Tucker and Bruce, 1995; Tucker *et al.*, 1997). However, these tests were carried out in pure culture conditions, against a select group of target basidiomycetes, not soft rot fungi. Developing a screening system based on non-sterile soil was necessary in order to test the antagonistic capacity of the potential biocontrol agent against one of the most aggressive environments in which wood can be placed during service. The use of non-sterile soil also provides a test medium containing a diverse range of micro-organisms, some of which are known decay organisms while others may be involved in the modification of the substrate, influencing the occurrence of decay and the pattern of colonisation (Gray, 1986).

The purpose of the preliminary screening carried out using *T. viride* isolate T60 was to assess the protective properties of the selected isolate, and in addition, examine the viability of the test system. The method was designed to be a screening system that is quick and easy to set up with an appropriate degree of replication and reproducibility and has proved to effectively meet the criteria in that decay in wood blocks could be observed after only a three-week incubation period (Table 2.1 (a)). Standard deviations in the results were relatively high but acceptable using the developed screening system, since statistical differences between test blocks and controls were still measurable.

Generally, the results from screening *Trichoderma* isolates as potential biocontrol agents to protect wood from decay fungi indicated that none of the isolates provided complete protection against non-sterile soil. However, the results did show a

statistically significant reduction in the amount of decay in wood blocks inoculated with T60 and IMI 49791. Although this protective effect was slight and only evident in blocks after the three-week incubation period, and does not therefore indicate successful biocontrol, it is a significant reduction as demonstrated by analysis of one-way ANOVA (Dunnets' test). It is clear that the critical period for determining the mechanisms influencing the observed reduction of decay in wood blocks treated with *Trichoderma* isolates, is the initial 3-week incubation. This is logical given the reduced size of the wood specimens used, and more frequent uplifts during this period may have indicated the source of the inhibition of decay fungi.

Redistributed soluble nutrients (RSN) have been shown to influence the rate of microbial colonisation and decay in wood and a relationship has been established between nitrogen content after burial and the rate of decay (Waite and King, 1979), indicating continuing invasion of micro-organisms from surrounding soil. It is possible therefore that the limited control achieved may have been due to prior removal of RSN by the *Trichoderma* isolates, delaying colonisation by decay fungi and slowing the decay process, though not stopping it completely.

Successful biocontrol of wood decay fungi by *Trichoderma* isolates has been demonstrated in the laboratory by a number of researchers (Srinivasan, 1993; Tucker and Bruce, 1995). However, it is now clear that the success of the biocontrol agent and the demonstration of antagonism are influenced by the substrate used to carry out the investigation. The effect of media composition on the antagonistic mechanisms of *Trichoderma* species' was investigated by Srinivasan *et al.* (1992a, b), in which the authors reported that substrate composition affected the production of lytic enzymes and volatile compounds. Further, Wheatley *et al.* (1997) also reported on the effect of media on the production of volatile organic compounds (VOCs) by *Trichoderma* spp.

The production of VOCs is regarded as one of mechanisms by which *Trichoderma* spp. repel other fungi. In addition, media composition can also affect the behaviour of target organisms, such as the inhibition of cellulase production by *C. globosum* in the presence of simple sugars (Eaton and Hale, 1993). The disparity between agar and wood as screening systems is highlighted by the failure of isolates selected on the basis of agar interaction studies to produce effective control in wood (Freitag and Morrell, 1990). The failure to correlate laboratory screening with field results suggest that the tests used to select potential biocontrol agents do not necessarily represent the natural environment of the fungi, especially in a substrate such as soil where a wide range of different biodeteriogens may be present. If biological control is to become an accepted method of protecting wood in ground contact from fungal degradation, the efficacy of potential biocontrol agents must ultimately be established using soil-wood block test systems or larger-scale specimens in field test systems. McNamara (1994) stated that soil block screening for wood preservatives using mono-cultures of basidiomycetes is virtually without merit in commercial wood preservation, and wood block-based screening systems are often considered to have limited suitability as a means for screening large numbers of potential biocontrol agents, in addition to being expensive and time-consuming. However, smaller samples may be more useful in evaluating potential biocontrol agents. Smaller wood blocks have a higher surface area to volume ratio and therefore expose greater areas of more easily colonised cross sections to test fungi. One problem identified in previous large-scale biological control tests was incomplete colonisation of wood by the biocontrol agent (Bruce and King, 1986b). The use of wood wafers rather than wood blocks has the potential to overcome limitations of time and also maximise colonisation (Freitag and Morrell, 1989). The use of smaller blocks provides rapid results; however, the degree of

reproducibility is reduced as the normal block-to-block fluctuations in moisture content and decay are over-emphasised. This could be resolved with the inclusion of more replicates in each treatment group used in the test system.

Bacterial cultures, and *Pseudomonas* spp. in particular, have been shown in laboratory tests to have an effective antagonistic effect against sapstain fungi (Benko, 1988, 1989; Croan 1996). The isolate used in this experiment was found as a contaminant on an agar plate of *T. pseudokoningii* (T64) cultured by a project student and its antagonistic properties were indicated by the observed inhibition of the *Trichoderma* mould fungus.

Agar plate interaction studies were carried out using the isolated *Pseudomonas* culture and 5 selected soft rot fungi. The results demonstrated a clear inhibitory effect on the growth of the fungi caused by the bacteria, and in some cases morphological changes were observed in the target organisms.

Although the outcome of the interaction study indicated an antagonistic effect against both *Trichoderma* and soft rot fungi, when the bacterial isolate was screened using the non-sterile soil system and wood blocks, no protective effect was evident (Table 2.5). Benko (1988, 1989) found that the progression from inhibition of sapstain fungi on agar plates to inhibition of fungi in wood blocks by *Pseudomonas* was successful, however the wood-based screening used in those experiments was agar-based. Differences in environment from agar plates to soil burial may have been too rigorous for the bacterial isolate to maintain its antagonistic properties i.e. there may have been insufficient nutrients available to the bacteria for production of soluble or volatile antibiotics. The influence of media type and composition has been shown to be crucial to the outcome of screening studies (Benko, 1989; Srinivasan *et al.*, 1992; Tucker *et al.*, 1996; Score, 1998) and it is possible that the observed inhibition

of the soft rot fungi in the interaction study was dependent on the nutrient composition of the agar medium.

Using a wood-based system, the bacterial isolate was tested against pure cultures of the selected soft rot fungi grown on malt extract agar plates (Tables 2.6, 2.7 and 2.8). The results were inconclusive due to a variety of reasons, primarily water-logging of the test blocks due to contact with the surface of the agar plate. If the moisture content of wood is too high, decay fungi cannot colonise or degrade the block. Excessive moisture may also inhibit colonisation of the potential control organism, however bacteria will preferentially colonise wood with high moisture contents (King, 1981). Other possible explanations for the outcome of the screening tests include questionable virulence of the soft rot organisms used, or more conceivably, a loss of antagonistic capabilities by the bacterial isolate. Due to the simpler nature of the bacterial genome, there is a higher probability of rapid mutation. If isolates are not sub-cultured and freeze-dried, they may become inactive or have their antagonistic properties severely reduced in a matter of months (Benko, 1988). Although the use of bacteria as a biocontrol agent for the prevention of sapstain has been reported (Benko, 1989), the potential loss of antagonism makes bacteria a less attractive option for protection against wood decay fungi where the requirement is for long-term prevention of colonisation and decay by basidiomycetes and soft rot fungi.

Chapter 3

Development of a Biological Control Delivery System

3.1 Introduction

Timber intended for use in ground contact situations is generally treated using chemical preservatives. Although preservatives such as creosote and CCA have been shown repeatedly to prolong the service period of ground contact timbers, premature failure of treated poles has nevertheless been observed (Hainey, 1992; Sinclair, 1995). Due to increasing awareness of the environmental impact of wood preservatives and the introduction of more stringent legislation over operations at treatment sites and the disposal of preservative treated wood, there has, over the last 25 years, been an upsurge of research into the potential of biological control as an alternative technology (Bruce, 1998). A critical aspect in the design of a biocontrol strategy is the delivery formulation and process used to apply the selected isolate to the substrate (Bruce, 1998). Post-treatment distribution and subsequent colonisation of the wood are important factors in the successful control of wood decay fungi, and a delivery method was selected that could potentially result in maximum distribution of the biocontrol agent. Although there are a number of examples in the literature regarding novel delivery of biological control agents (Bruce and King, 1986a; Schoeman *et al.,* 1994), this project utilises equipment currently used in the timber preservation industry. A pilot preservation plant was used to apply fungal spores to wood in a vacuum-pressure impregnation process, a novel delivery system for applying a biocontrol agent. The use and adaptation of current industry processes is designed to make this delivery system, and thus the use of fungal spores as biocontrol agents, more practicable to the chemical preservation industry.

Biological control agents for the protection of wood and wood products need to be effective over long service periods without degrading the timber structure. These requirements indicate a need for the development of delivery formulations containing extraneous nutrients essential to the growth of the biocontrol agent (Bruce,

1998), for example nitrogen, an element in which wood is particularly low. However, the inclusion of an additional nutrient source may also serve to accelerate colonisation of decay organisms such as soft rot fungi, which require an extraneous nitrogen source (Butcher and Drysdale, 1974). An alternative solution may be the use of a nutritionally non-exacting, non-degradative wood coloniser such as *Trichoderma* spp.

Trichoderma is currently the most extensively researched biocontrol fungus in the field of forest products protection and a number of isolates have been found to be particularly effective against certain wood decay fungi (Highley & Ricard, 1988; Bruce and Highley, 1991). Tucker *et al.* (1997) have shown that certain isolates of *Trichoderma* can protect wood against basidiomycete decay fungi. These results demonstrated that one particular *Trichoderma viride* isolate (T60), was totally effective in protecting wood blocks from the decay action of a wide range of selected basidiomycetes. These results, using modified versions of American (AWPA M10, AWPA 1977) and European (EN 113, CEN 1986) standard test methods as well as a soil burial test system, demonstrated that this *Trichoderma viride* isolate (T60), was totally effective in protecting wood from the decay action of the selected basidiomycetes. Based on results reported by Tucker *et al.* (1997) and those obtained using the preliminary screening system described in chapter 2, *T. viride* isolate T60 was selected as a potential biocontrol agent for use in field and fungal cellar tests.

Novel wood preservative formulations need to be tested within recognized guidelines such as European Standards, and in a manner relevant to the proposed end use of the treated wood. According to European Standard EN 599, "Durability of wood and derived materials – performance of wood preservatives as determined by biological tests" (CEN 1992), chemical preservatives applied using a penetrating process for use in Hazard Class 4 (ground contact situations) are required to be tested

in ground contact, using European Standard EN 252, "Field test method for determining the relative protective effectiveness of wood preservatives in ground contact" (CEN, 1989). In order to fully assess the efficacy of a potential biocontrol agent designed for use with ground contact timbers and applied using a penetrative process, a field trial was designed to test the protective capacity of the selected biocontrol isolate. Soil contact is commonly regarded as the most damaging environment in which wood can be placed in service (King, 1981). Field trials for the biocontrol of deterioration in timber in ground contact are therefore essential to establish the full validity of biological control for wood protection.

This chapter describes the planning and implementation of large-scale field and fungal cellar tests, using wooden stakes pressure impregnated with *T. viride* spores. European Standard EN 252 (CEN, 1989) was used as a framework for the design of this field trial. Parameters such as wood type, stake size, orientation, reference preservative and burial pattern were selected from the standard.

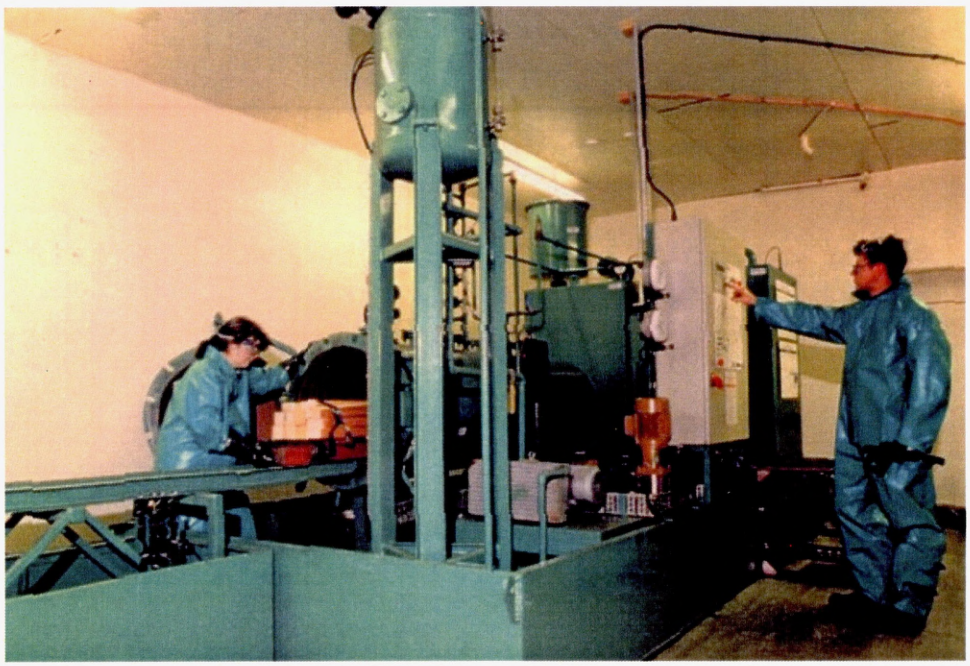


Figure 3.1: Pilot-scale preservation cylinder used for pressure impregnation of wood

Materials and methods

3.2.1 Wood conditioning

One hundred and twenty stakes each of Scots pine (*Pinus sylvestris* (L)) and Sitka spruce (*Picea sitchensis* (Bong).Carr) measuring 500 mm longitudinal x 50 mm radial x 25 mm tangential were dried at 40°C for 2 weeks then allowed to condition at ambient temperature (10-12°C) for 6 weeks. During this time, the moisture contents of a representative sample group of the stakes (40 each of spruce and pine) were measured every 7 days using a Diagnostic Timbermaster moisture meter (Protimeter plc., Bucks., U.K.). Measurements were taken from 3 positions on one longitudinal face of the stakes. Moisture measurements were recorded for all stakes prior to treatment. It must be noted at this point that moisture measurements taken using electrical conductivity devices such as moisture meters are not accurate moisture contents, particularly above 30% (as opposed to moisture contents measured using dry weight analysis). As such, moisture meters are used simply to give an indication of whether wood is above or below fibre saturation point.

3.2.2 Preliminary laboratory-based experiments

Culture maintenance and media preparation were carried out as described in Appendix A: Culture Methods and Isolation. Chemical suppliers are detailed in Appendix B: Chemicals, Reagents and Suppliers.

3.2.2.1 Bench-top pressure impregnation

Preliminary experiments to assess the suitability of pressure impregnation for the application of fungal spores and to determine their penetration into wood blocks were undertaken using a bench-top pressure impregnation system as described in

AWPA M10 (1977). Five replicate blocks each of spruce and pine, measuring 100mm longitudinal x 50mm radial x 25mm tangential, were cut from conditioned stakes using a bandsaw. The wood blocks were then treated with a T60 spore suspension at a concentration of 10^6 spores ml^{-1} and a similar number of each species were treated with sterile water as controls. Each wood block was weighed before and after impregnation to measure the volume of fluid uptake of both the spore suspension and water. The T60-treated blocks were sectioned longitudinally and slivers of wood were removed from the newly-cut surfaces 5-10mm in from the longitudinal edges and from the centre of the radial face, with sampling sites approximately 1cm apart. These wood slivers taken from various depths into the block were plated out onto 3% malt extract agar (Figure 3.2, parts a, b and c) and incubated at 25°C for 2 weeks; plates were viewed every 2 days and growth was recorded. *Trichoderma* growth established that spores survived pressure treatment and gave an indication of the distribution of the spores following treatment.

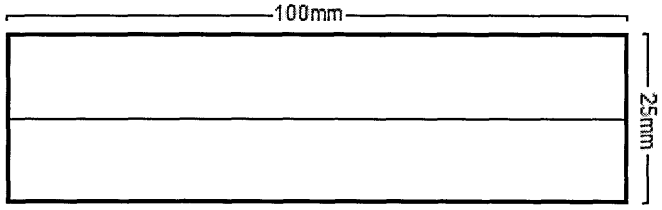


Figure 3.2 (a): The longitudinal division of treated wood blocks

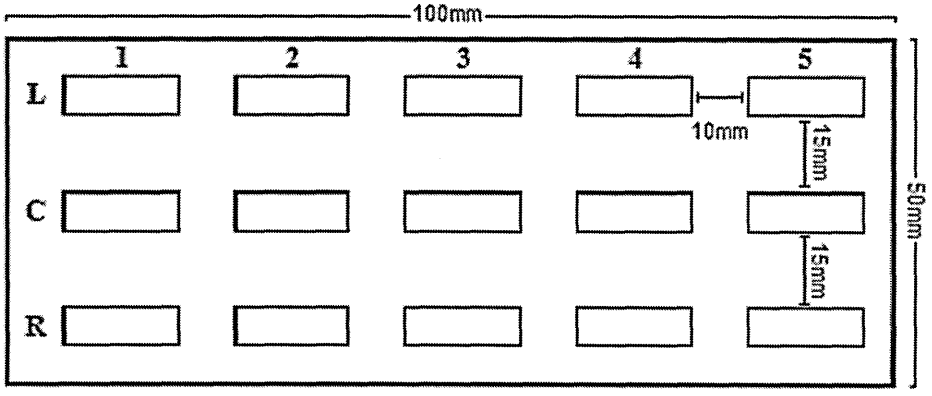


Figure 3.2 (b): The position of samples taken from within treated blocks

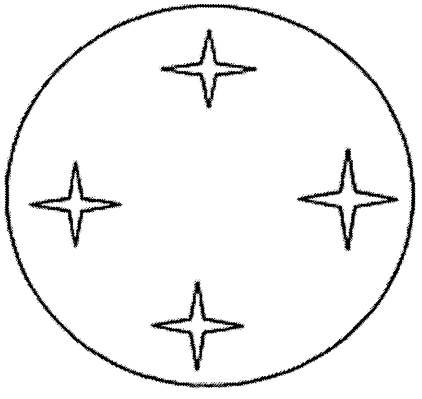


Figure 3.2 (c): Wood slivers plated onto agar plates

Figure 3.2: Isolation of *Trichoderma* from wood following bench-top pressure impregnation of T60 spores

3.2.2.2 Effect of water sterilisation salts on spore viability

Due to the large volume of sterile water required to prepare the T60 spore suspension for the process of pressure impregnation, commercially available water sterilisation tablets (Puritabs Maxi (sodium dichloro-s-triazinetrione 425mg)) were selected as the most suitable method for sterilisation. In order to identify any major adverse effects of the water sterilisation tablets on the growth of *Trichoderma* spores, serial dilution plates were set up using spore suspensions prepared in autoclaved sterile, distilled water; non-sterile tap water taken from a sink in the laboratory; and tap water purified by the addition of water purification tablets at the recommended dosage of 34 mg L^{-1}.

Spore suspensions were produced using each of the three differently treated types of water and diluted to contain 1 x 10^5 spores/ml. A series of dilutions was then prepared to give the spore concentrations detailed in Figure 3.3 below.

Serial dilutions	Spores/ml
-	1 x 10^5
1:10	1 x 10^4
1:100	1 x 10^3
1:1000	100

Figure 3.3: Spore concentrations used to assess the effect of water purification tablets on *Trichoderma viride* (T60) growth.

Twelve 5% malt extract agar plates were inoculated by pipetting 1 ml of the appropriate spore suspension onto the surface of the agar, which was then spread over the surface of the plate using a flame-sterilised glass spreader. The plates were incubated at 25°C for 3 days before the number of colonies on each plate was counted and recorded.

3.2.2.3 Preparation of spore inocula

Five hundred 3% malt extract agar plates were inoculated with *Trichoderma viride* isolate T60 using either a sterile inoculating loop or 6mm cores. The plates were incubated at room temperature for 4 weeks until profuse spore growth was apparent. Spore suspensions were prepared using 19 litres of autoclaved sterile, distilled water. The approximate mean concentration of spores per ml of each suspension was counted using a haemocytometer, and an average spore concentration of 7.4×10^8 spores per ml was calculated prior to dilution.

3.2.2.4 Sterilisation of water for spore suspension

Approximately 900 litres of tap water was sterilised using purification tablets as described above prior to addition of the concentrated spore suspension. This gave a final treating concentration of around 8×10^5 spores ml^{-1}.

3.2.3 Impregnation of stakes for field and fungal cellar exposure

3.2.3.1 Selection of pressure cycles

Having established that the spores survived bench-top pressure impregnation, the appropriate pressure treatment cycle for use in the preservation plant (pilot model, Hicksons U.K., Castleford, U.K..) was determined by "treating" 5 replicate spruce and pine stakes using water under each of the recommended cycles (BS 4072 (BSI, 1987), BS 5589 (BSI, 1989)) for both wood types (see Figure 3.4). This was undertaken to assess solution uptake and to determine the extent of stake-to-stake variations.

Pressure cycle	Specifications	
P3 (recommended for pine)	**vacuum**	600 mmHg for 30 minutes
	pressure	12.8 Kg/m^3 for 90 minutes
	vacuum	600 mmHg for 30 minutes
P4 (recommended for spruce)	**vacuum**	600 mmHg for 60 minutes
	pressure	12.8 Kg/m^3 for 180 minutes
	vacuum	600 mmHg for 60 minutes

Figure 3.4: Details of pressure cycles used to treat wooden stakes in a pilot preservation plant

3.2.3.2 Treatment of wood with spores

The treatment cycle finally selected for both wood types was P3, recommended for pine for use in ground contact (BS 4072 (BSI, 1987), BS 5589 (BSI, 1989)): an initial vacuum of 600 mmHg for 30 minutes followed by a 90 minute pressure period at 12.8 Kg/cm^2. Forty stakes each of Scots pine and Sitka spruce were pressure-impregnated with the fungal spore solution prepared. No final vacuum was drawn on the spore treatment cycle to avoid removal of any spores from the wood surface.

3.2.3.3 Treatment of wood with CCA

Cycle P3 was also used to treat 40 spruce and 40 pine stakes with a reference chemical preservative (Tanalith CCA - 3%, Hicksons, U.K.) for use as preservative-treated controls. A final vacuum was drawn to remove as much free CCA from the wood as possible. Field trial guidelines laid out in EN 252 recommend the application of a 0.5% CCA solution for reference preservative control stakes. However, the recommended commercial application of 3% was used in this field trial to ensure adequate protection of wood used for fungal cellar testing.

3.2.4 Conditioning of wood post-treatment

Stakes treated with T60 spores were left in the treatment cylinder for 30 minutes to drip, then removed from the cylinder and weighed. The T60-treated stakes were stacked in a plastic tray and loosely covered with a sheet of perspex. The preservative-treated stakes were removed from the cylinder only after CCA solution was no longer dripping from the wood. These stakes were also placed in a plastic tray but were left uncovered.

Trichoderma-treated stakes were conditioned for a period of 16 weeks (to allow sufficient germination of the biocontrol fungus) and the CCA-treated stakes for a period of 5 weeks. After this time, the moisture contents of the treated stakes (10 from each treatment, 5 of each wood species) were recorded prior to burial in the field and fungal cellar.

3.3 Results

3.3.1 Wood conditioning

Day no.	Wood moisture content (%)	
	Sitka spruce	Scots pine
0	11.9 (1.0)	9.7 (0.9)
7	10.9 (0.9)	8.9 (0.8)
14	11.7 (0.7)	9.0 (0.9)
21	12.2 (0.9)	9.8 (0.6)
28	12.3 (0.6)	10.5 (1.0)
35	12.1 (0.5)	10.8 (0.8)
42	12.3 (0.9)	11.0 (1.2)

Table 3.1: Mean moisture contents of wood stakes during pre-treatment conditioning period. Means are calculated from the moisture contents of 40 stakes each of Scots pine and Sitka spruce measured at 7-day intervals. Figures in parentheses denote standard deviations.

The results in Table 3.1 show an initial drop in the moisture contents of the sample stakes after removal from kilns, then a gradual increase throughout the conditioning period. Mean moisture contents for both wood species have low standard deviation values, and the results also show that the Sitka spruce stakes had a slightly higher moisture content than the Scots pine samples. At the end of the conditioning and monitoring period, stakes were still well below the recommended maximum pre-treatment moisture content of 28%.

3.3.2 Preliminary laboratory-based experiments

3.3.2.1 (a) Bench-top pressure impregnation

Uptake	Spruce + T60	Spruce + H_2O	Pine + T60	Pine + H_2O
Kg/m^3	641.0 (29.2)	778.8 (68.7)	592.4 (31.3)	696.6 (20.6)
Weight/weight (g)	2.01 (0.12)	2.39 (0.21)	1.24 (0.09)	1.43 (0.15)

Table 3.2: Mean uptake of T60 spores and water by wood blocks following pressure impregnation. Five replicate blocks were treated in each category. Figures in parentheses denote standard deviations.

As the results presented in Table 3.2 demonstrate, there has been consistent uptake in all the treatment groups. However, there were higher uptakes in blocks of both wood species treated with water. This may be due to particulate matter in the spore solution blocking apertures in wood. There was also a clear demonstration of significantly higher uptakes of both treatment solutions in spruce blocks than in pine, possibly due to the anatomical differences between the wood species such as density and void volume.

3.3.2.1 (b) Distribution of *Trichoderma* spores following bench-top pressure impregnation

wood type	position	Section number				
		1	2	3	4	5
Scots pine	left	100	50	75	0	100
	centre	100	25	0	25	100
	right	100	25	25	50	100
Sitka spruce	left	100	25	50	50	100
	centre	100	25	50	0	100
	right	100	100	75	75	100

Table 3.3: Percentage of samples showing *Trichoderma* growth on malt extract agar up to 14 days following bench-top pressure impregnation of wood blocks with T60 spores.

Samples excised from the outside sections of T60-treated wood yielded growth from all blocks sampled (see Table 3.3). This implies a higher saturation of spores in first 5-10 mm of the wood as well as a degree of surface coating, although blocks were surface sterilised during sampling to reduce this possibility. The frequency of re-isolation from spruce samples was slightly higher in many sample points than in the equivalent pine samples. This ties in with the recorded uptake results shown in Table 3.2, where the uptakes per volume and per gram of dry weight are both higher for spruce blocks than for pine blocks.

3.3.2.2 Effect of water sterilisation salts on spore viability

Dilution	Spores/ml	Sterile, distilled water		Puritab-treated water		Non-sterile tap water	
		colonies	%	colonies	%	colonies	%
-	1×10^5	TNC	-	TNC	-	**x**	-
1:10	1×10^4	TNC	-	TNC	-	**x**	-
1:100	1×10^3	417	100	322	77.22	**x**	-
1:1000	100	72	100	51	70.83	14*	19.44

Table 3.4: Colonies of T60 growth on malt extract agar plates inoculated with serial dilutions of T60 spores prepared in sterile distilled water, untreated tap water or tap water containing water purification salts.

Key

TNC = too numerous to count; **x** = plate overgrown with bacterial colonies;

* = tentative count (bacterial growth obscuring *Trichoderma* colonies)

Table 3.4 shows a significant reduction in the growth of *Trichoderma viride* isolate T60 colonies on agar plates inoculated with a T60 spore suspension prepared using non-sterile tap water. This is shown in comparison to plates prepared using sterile distilled water and tap water sterilised using Puritabs. The non-sterile tap water plates were covered in bacterial colonies, which made enumerating the presence of *Trichoderma* extremely difficult. Sterile distilled water was used as a measure of the maximum quantifiable growth, therefore theoretically 100%. Although there was a drop in the viability of T60 spores when the spore solution was made up in Puritab-treated tap water, this reduction was not as extreme as that caused by the use of untreated tap water and around 75% of spores were still viable.

3.3.2.3 Selection of pressure cycles

Pressure cycle	Uptake	Scots pine	Sitka spruce
P3	**Kg/m^3**	645.02 (15.55)	649.92 (68.59)
	Weight/weight (g)	1.35 (0.06)	2.00 (0.24)
P4	**Kg/m^3**	577.01 (134.86)	652.56 (107.11)
	Weight/weight (g)	1.33 (0.31)	2.14 (0.26)

Table 3.5: Mean uptake (Kg/m^3) of water by Scots pine and Sitka spruce stakes following pressure impregnation to determine the optimum treatment cycle. Mean is calculated from 5 replicate stakes for each category. Figures in parentheses represent standard deviations.

Although cycle P4 is the recommended process for use with spruce wood species, the difference in uptake for spruce stakes treated using each cycle is negligible, particularly when the standard deviation values are taken into consideration. Table 3.5 shows however, that there is far more variation of treatment and resultant uptake for both wood types when cycle P4 was used to treat spruce and pine stakes. In addition to this, the uptakes recorded in pine stakes treated in cycle P4 were significantly lower than those achieved using cycle P3, the recommended treatment cycle for use with pine wood species. It was therefore decided that for the final treatment of field trial stakes with a *Trichoderma* spore suspension, cycle P3 would be used.

3.3.3 Impregnation of stakes for field and fungal cellar exposure

3.3.3.1 Treatment of wood with spores

Uptake	Scots pine	Sitka spruce
Kg/m^3	679.26 (27.9)	596.56 (41.6)
Weight/weight (g)	1.29 (0.48)	1.80 (0.42)

Table 3.6 Mean uptake of T60 spore solution in Scots pine and Sitka spruce stakes treated using a pilot pressure impregnation plant.

Following pressure impregnation of wood with spores, uptakes were calculated on both a volume basis and a weight for weight basis. The results in Table 3.6 show that, as with bench-top pressure impregnation of spores, spruce stakes have a higher uptake of spore solution per gram of dry weight than pine stakes. However, when the results for uptake per volume are assessed, pine stakes appear to have a slightly higher uptake of T60 spore solution than spruce stakes. This may be explained by again referring to anatomical differences between the wood species, as pine is more dense (and thus heavier) than spruce.

3.3.3.2 Treatment of wood with CCA

Uptake	Scots pine	Sitka spruce
Kg/m^3 (Soln)	618.89 (12.5)	576.65 (51.2)
Kg/m^3 (Salts)	18.85 (5.3)	17.56 (4.8)
Weight/weight (g)	0.99 (0.02)	1.58 (0.14)

Table 3.7 Mean uptake of CCA solution in Scots pine and Sitka spruce stakes treated using a pilot pressure impregnation plant.

As with the results of the application of T60 spore solution, CCA uptake differs between pine and spruce according to the method used to determine uptake. The results for pine stakes in Table 3.7 show a higher uptake of both CCA solution and CCA salts per volume than those calculated for spruce. Uptakes calculated per gram of dry weight indicate that on a weight for weight basis, spruce stakes have a higher uptake of CCA than pine stakes, although the overall level of uptake was slightly more variable in spruce. This variability was expected, as it was indicated in the preliminary assessments of pressure cycle selection. The increased concentration of the CCA solution applied is evident in these results, indicated by the elevated uptakes.

3.3.4 Pre-planting moisture contents

Treatment group	Site	Scots pine	Sitka spruce
CCA	**field**	19.1 (0.82)	43.2 (8.91)
	cellar	13.1 (0.63)	17.8 (0.85)
T60	**field**	28.0 (1.41)	33.0 (2.12)
	cellar	20.4 (0.21)	24.1 (0.28)
Untreated	**field**	10.7 (0.17)	11.3 (0.24)
	cellar	11.0 (0.14)	11.8 (0.16)

Table 3.8 Final mean moisture measurements of stakes recorded prior to planting at the field site or in the fungal cellar. Means are calculated from 5 stakes from each group. Figures in parentheses represent standard deviations.

The results in Table 3.8 show that Scots pine stakes have a consistently lower mean moisture content than Sitka spruce stakes, regardless of treatment. Stakes planted in the fungal cellar were conditioned for a longer period of time than field stakes (16 weeks and 10 weeks respectively), due to a delay before planting and this is reflected in the significantly lower moistures in the CCA- and T60-treated stakes for this category. *Trichoderma*-treated stakes have stayed wetter for longer i.e. the mean moisture contents were still over 20% before cellar burial. This is thought to be a result of the difference in conditioning methods for the biological control agent in comparison to the CCA-treated stakes. The CCA-treated Sitka spruce stakes planted in the field had a relatively high moisture content in contrast with the other CCA-treated stakes, however this may have been either the result of the on-going evaporation of moisture from the treated wood or perhaps the effect of CCA salts on the electrical conductivity meter used to record the moisture contents. Figure 3.5 shows T60-treated stakes of both wood species, photographed prior to planting in the

fungal cellar; *Trichoderma* spores can clearly be seen on the wood surfaces and it was observed that pine stakes displayed more growth than spruce following conditioning.

Figure 3.5 (a): Pine stakes

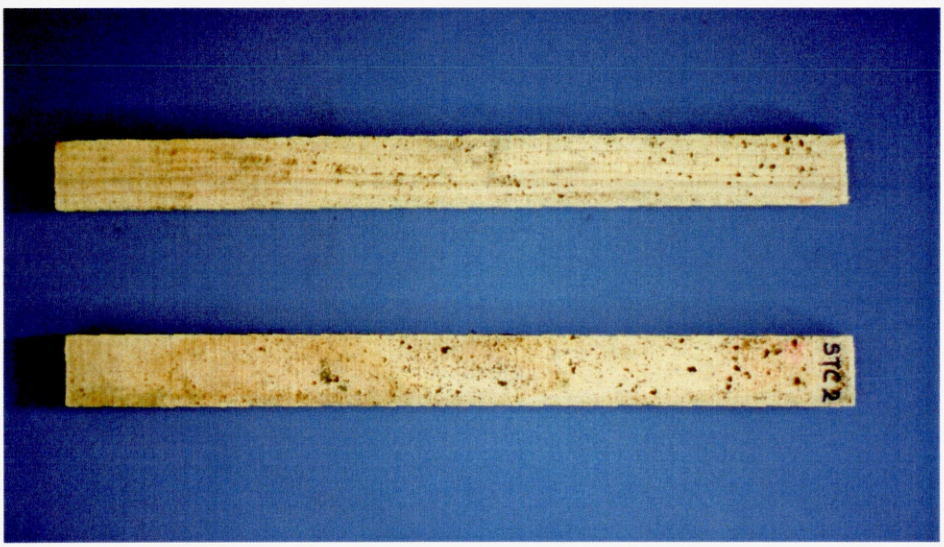


Figure 3.5 (b): Spruce stakes

Figure 3.5: Spruce and pine stakes showing surface growth of *Trichoderma* following pressure impregnation with T60 spores and incubation

3.4 Discussion

The means by which a biological control agent is applied i.e. the delivery system, is a critical factor in the success of any biocontrol strategy. In agriculture, delivery of biocontrol agents is similar to the methods used to apply chemical biocides. However, wood preservatives are generally applied by pressure processes under high temperatures (Bruce, 1998). The ability of fungal biological control agents such as *Trichoderma* spp. to colonise a substrate by growth has been at the root of research into a variety of highly original application methods for the bioprotection of wood, particularly freshly felled timber (Schoeman *et al.*, 1994). It is important to remember however, that short-term (i.e. post-harvest) and remedial treatments do not have the same requirements as long-term bioprotection of wood in ground contact situations. Therefore, it may be necessary to develop and adapt delivery systems utilising processes used for traditional chemical preservatives (Bruce, 1998).

This chapter focused on the development and use of a pressure impregnation system to apply spores of *Trichoderma viride* isolate T60 to wooden stakes for use in a ground contact field trial and fungal cellar test. The ability of fungal spores to survive pressure impregnation using bench-top treatment equipment was reported by Tucker *et al.* (1997) and Brown and Bruce (1998) described the pressure-impregnation application of fungal spores to wood using a pilot preservation plant, with treatment regimes equivalent to those used in industry. Subsequent germination of the applied biocontrol agent established that biological control strategies can successfully utilise existing industry processes (Brown and Bruce, 1998, 1999).

The pre-treatment conditioning of wood for the field trial was carried out to ensure an even moisture content throughout the stakes (individually and as a group) and to allow the stakes to achieve equilibrium with the ambient conditions within the treatment area – the room in which the pilot preservation plant was situated, also used

for conditioning the stakes before and after treatment. There were no significant changes in the moisture contents of the monitored stakes during the conditioning period; after an initial drop in moisture immediately following removal of the wood from the kilns, there was a gradual increase in the moisture contents of both wood species as the stakes acclimatised to the treatment room.

The preliminary, laboratory-based experiments carried out to establish the feasibility of spore impregnation in terms of uptake and subsequent distribution of a proposed biocontrol agent showed that blocks impregnated with a T60 spore solution had lower uptakes per volume than blocks impregnated with water alone. This may be due to the presence of particulate matter in the form of spores and other fungal fragments in the treatment solution, leading to an overall increase in "viscosity" of the solution. This would reduce the overall uptake as these particles and other debris block pits and other avenues of entry into the wood (e.g. ray tissues etc.). However, although the reduction in uptake is noticeable, it is not severe enough to cause a major problem with regard to the pressure impregnation of spores.

Uptake per volume is higher in the spruce blocks than the pine for both water and the spore solution. The most apparent explanation for this is the lower density and higher void volume of spruce in comparison with pine, resulting in more space for water molecules to enter the blocks.

In the experiment aimed at determining the distribution of spores in wood following pressure impregnation, *Trichoderma* spp. was re-isolated from wood samples taken from various positions within the blocks (see Figure 3.2). Samples were excised almost immediately following treatment i.e. there was no post-treatment conditioning period. Therefore there was very little time for the gradual movement of spores within the wood (e.g. through "capillary action" etc.), or for germination of spores and extension of hyphae. There was still significant penetration but it is not

known how much of this may have been due to spores entering via end grain penetration of the blocks as opposed to via the pits and other apertures from the radial or tangential wood surfaces. If a significant amount of spore uptake is via end grain penetration of the wood, this will have a critical effect on the success of subsequent treatment efficacy of larger field trial stakes. It also illustrates that the difference in wood block sizes used in laboratory tests and field trial tests can make the direct correlation of preliminary testing of treatment efficacy results with those of the field trial difficult, due to the difference in surface area to volume ratio in the laboratory blocks compared to that of the full-size field trial stakes.

As expected, the most successful re-isolation was achieved from samples taken from the longitudinal aspect of the blocks, where 100 % of samples yielded growth of *Trichoderma*. There appears to be better impregnation and uptake of spores in spruce blocks compared with pine, as suggested by results of uptakes per volume (Table 3.2). These results suggest that spores have successfully been pressure impregnated deep into the wood, based on the frequency of re-isolation of *Trichoderma* from treated blocks. Another aim of the experiment was to demonstrate spore viability following pressure impregnation and these results showed that spores can survive the extreme pressure conditions involved in the method of application.

Due to the relatively low numbers of stakes being treated in this instance and the practicality in producing only one spore suspension of such volume, representative stakes of both wood species were "pressure impregnated" with water on cycles recommended for sawnwood of each species, Scots pine and Sitka spruce. The difference between the cycles takes into account the refractory nature of spruce by employing longer vacuum and pressure periods. However, the results show that while there was relatively little difference in uptakes between cycles P3 and P4 for spruce (with P4 being the cycle recommended for spruce species), pine stakes showed

a reduced mean uptake per volume when cycle P4 was used. Overall, cycle P3 was selected as being appropriate for both wood species, based primarily on lower variability of mean uptakes as indicated by the lower standard deviation (see Table 3.5).

Uptakes of T60 spore solution and CCA by field trial pine and spruce stakes indicated that both wood species had a higher uptake of T60 spores than CCA per gram of dry weight, believed to be due to the differences in treatment solution properties. Results presented in Tables 3.6 and 3.7 also indicate that while uptakes of both solutions appeared to be slightly higher in pine stakes than in spruce stakes when viewed per volume, this is transposed when results are calculated on a weight for weight basis. This may be a result of anatomical differences between wood species, as pine is heavier and more dense than spruce, therefore weight increases in spruce will be exaggerated in comparison to pine.

In order to carry out the pressure impregnation process, a volume of around 900 litres of water (or water-based preservative) was required. As it was not feasible to autoclave and transfer such a large volume of water without risking external contamination, and based on laboratory experiments determining the suitability of untreated tap water, one alternative was the use of commercially available water-sterilising tablets (see 3.2.2.2). Serial dilutions carried out to compare the viability of spores suspended in Puritab-treated water with those in autoclaved distilled water showed only a 25% reduction in viability. In contrast, the viability of spores contained in untreated tap water was reduced by around 80% when compared with sterile distilled water. In some cases the presence of bacteria and other contaminants made it impossible to successfully detect or count T60 colonies. None of these contaminants appeared on plates spread with Puritab-treated spore suspensions. However, the numbers of germinating spores were slightly fewer than on plates

inoculated with conventionally sterilised water. While it is true that many of the contaminants present in tap water may have little or no effect on the establishment of *Trichoderma* as a biocontrol agent in wood, the relatively low decrease in spore viability made the use of water-sterilising salts a viable option in the development of a successful delivery formulation.

Untreated control stakes were kept in the same room as treated wood throughout the period during which the treated stakes were allowed to condition, therefore any differences in moisture contents of untreated stakes can be attributed directly to the effects/influence of external factors such as temperature and weather on the conditioning area. As before, spruce stakes maintained a marginally higher mean moisture content than pine stakes.

The primary reasons for post-treatment conditioning were to allow the fixation of CCA salts in the preservative-treated stakes, and to promote spore germination in the T60-treated wood. The importance of salt fixation in CCA-treated wood is known, although the mechanisms behind the fixation process have been described as "abstract and non-defined" (Christensen, 1990: cited in Hainey, 1992). Research into the permanence of CCA in wood has indicated that sufficient time is required post-treatment for the formation of preservative complexes within the lumen and the wood cell wall. The reasoning behind promoting spore germination prior to in-service use of wood treated with a biocontrol agent is to allow the antagonist to become established by forming a fungal "network" throughout the treated substrate, reducing the incidence of unprotected areas of wood before the stakes are exposed to potential biodeterioration.

Despite the apparently anomalous elevated moisture content in CCA-treated spruce planted at the field site, there is a distinct pattern visible in the moisture contents of the stakes (as recorded prior to planting), although it must again be noted

at this point that electrical conductivity devices are not accurate above 30% moisture content. Spruce stakes have a higher mean moisture content than pine, attributable to slower drying of this timber type due to the anatomical features of the wood (Hainey, 1992); cellar stakes were conditioned for longer than the field stakes due to staggered planting, explaining the significantly lower moisture contents of treated stakes prior to cellar burial.

The moisture contents of T60-treated stakes were higher than both CCA-treated and untreated stakes (with the previously mentioned exception). There are a number of probable explanations for this, the most significant factor being that no final vacuum was drawn at the end of the T60 treatment cycle to prevent potential removal of spores on or near the surface. In addition to this, stakes treated with the biocontrol agent were kept covered during post-treatment conditioning to elevate temperature and relative humidity, aspects encouraging spore germination. The importance of moisture levels to spore germination was noted by Philp (1998), who reported that *Trichoderma* spores did not germinate and colonise through Sitka spruce and Scots pine logs when the biocontrol agent was applied as a surface coating or as a pellet because the moisture content of the wood was too low. Another possible factor contributing to the increased moisture content of T60-treated stakes is the effect of the biocontrol agent itself through respiration, retaining moisture as an aid to survival and growth.

The effects of CCA salts on electrical conductivity and wood moisture content are important considerations when moisture contents are measured using electrical conductivity moisture metres. CCA-treated stakes have been reported previously to have a lower moisture content than untreated wood (Green, 1988) as a result of chromium (VI) molecules binding to hydroxyl groups within the wood cell wall, reducing the number of binding sites available for water and expelling moisture

already present in the structures. This effect may also explain (in part) the elevated moisture content of CCA-treated spruce stakes planted at the field site. As CCA salts fix, water is repelled and must travel to the wood surface to evaporate. If the CCA took longer to fix in spruce than in pine, the high moisture contents recorded may have been caused by the accumulation of water at the surface of the stakes, as part of the fixation process.

Other means of applying biocontrol agents to substrates such as wood include painting, spraying and dipping. The first two methods may be considered to be more appropriate for the remedial treatment of wood still in-service e.g. structural timbers in historic buildings, and there has been documented use of these methods in such cases (Score, 1998). Dipping has also been used for the application of biocontrol agents (Rosner *et al.*, 1998; Tucker *et al.*, 1998). However, for the treatment of large volumes of sawn wood, pressure impregnation appears to be the most logical and industry-compatible system, particularly when full penetration and distribution of applied biocontrol agents is required.

There are a number of novel inoculation systems that have been used to apply biocontrol agents to wood, including the production of BINAB pellets, then inserted into small core-holes drilled in telegraph poles (Bruce and King, 1986a, b; Philp, 1998), and the application of *Trichoderma* spores in chainsaw oil during harvesting of trees (Schoeman *et al.*, 1994). However, due to the many and varied applications of wood in service to which biocontrol may be applied, delivery systems and formulations must be developed with the type of wood product and the end-use of that particular product in mind.

There are several reasons for selecting a spore suspension as a delivery formulation, principally the need for a liquid treatment medium for use in pressure

impregnation. In addition to this, conidiospores ("spores") are one of the fungal reproductive components likely to survive the harsh conditions involved in pressure impregnation processes. Hyphal fragments are sensitive to environmental stresses (Hjeljord and Tronsmo, 1996), and were thought to be too fragile to withstand large-scale preparation and treatment. Conidiospores are easily produced in large amounts, and are physiologically hardy. Although conidia are sensitive to environmental conditions and may encounter soil fungistasis (Hjeljord and Tronsmo, 1996), in this project this was less relevant as the wood would provide the extraneous nutrients required to stimulate germination. The size of a conidiospore in comparison with any apertures such as pits in sawnwood make it possible that, in combination with the extremes of pressure used in the process, spores can be deposited to a significant depth within the wood. When the spores subsequently germinate in the wood, they can form a type of "mesh" throughout the substrate, leaving fewer pockets of unprotected wood.

Large-scale production of any proposed biocontrol agent is also an important factor in the development of a biocontrol strategy. Spores have the capacity to be "grown" and harvested on a much larger scale than other fungal structures (Hjeljord and Tronsmo, 1996).

The development of a delivery formulation of T60 spores suspended in water was based in part on the fact that *Trichoderma* spp. are natural non-degradative colonisers of wood. As nutritionally non-exacting organisms, *Trichoderma* are able to colonise wood and initiate spore germination in the absence of extraneous nutrient sources. In addition to this, water provides a simple, safe and economical treatment solution that would have less impact on the local microbial ecology than a nutrient-rich treatment solution when the treated wood is placed in ground contact. Results presented in Chapter 2 (Table 2.5) suggested that the presence of nutrient broth in

wood blocks exposed to soil burial may have exaggerated invasion by soil-based wood decay micro-organisms. The use of water as a treatment medium also ensures that natural competition for nutrients, a possible biological control mechanism of *Trichoderma* spp., will not be adversely affected.

In order for biological control to succeed as a viable alternative to traditional chemical preservation, particularly with regard to long-term protection of ground contact timber, a number of aspects of the delivery system need to be investigated in detail. The results presented in this chapter give a clear indication that it is possible to use pressure impregnation equipment designed for the delivery of chemical preservatives to treat wood with a biological control agent, with relatively few alterations required to the processes involved.

Chapter 4

Field Trial and Fungal Cellar Testing of a Biological Control Agent

4.1 Introduction

Biological control, most simply defined as the use of a naturally-occurring antagonist to control or eradicate a pest - has been proposed as an alternative to chemical control in a number of situations including agriculture and forestry (Bruce, 1998). Although the application of biocontrol is now well-established in agriculture, there are a number of factors influencing the selection and use of biological control agents in forest products technology. Aside from formulation considerations, biocontrol agents developed for the protection of wood must show acceptable long-term protection without the need for reapplication, in order to compete with or replace chemical wood preservatives (Sinclair, 1995). Most agricultural applications of biological control require protection for one growing season, i.e. one year or less (Bruce, 1998). With a few exceptions, such as post-harvest prevention of sapstain colonisation in freshly felled timber (Schoeman *et al.*, 1994), biological control of forest products (e.g. sawn wood) needs to be compatible with the long service period expected of wood intended for purposes such as ground contact. In addition to this, the biological control agent must be shown not to cause significant damage to the structure of the wood.

Chemicals developed for the protection of wood and wood products against degradative agents such as insects and fungi are generally assessed by following a standard screening procedure, for example, EN 113 (CEN, 1996); AWPA M10-77 (AWPA, 1977) (Tucker *et al.*, 1997). Laboratory based assessments are routinely used to select potential wood preservatives from a wider range of possible formulations (Archer *et al.*, 1993). Once a suitable agent has been selected it is further tested in a field trial, in which the treated wood is exposed to degradative agents in a manner similar to the intended use of the wood or wood products. Field tests for chemical preservatives are standardised according to the service use of the

timber and the major pests associated with those situations. Chemical wood preservatives designed for the protection of wood in ground contact are tested according to European Standard EN 252 "Field test method for determining the relative effectiveness of a wood preservative in ground contact" (CEN, 1989), as stipulated in European Standard EN 599, "Durability of wood and derived materials – performance of wood preservatives as determined by biological tests." (CEN, 1992). This standard specifies the testing requirements that preservatives formulated for use with wood categorised as Hazard Class 4 must be assessed by. Wooden stakes are treated with the new preservative agent and buried to half their length in soil alongside stakes treated with a reference preservative of known effectiveness to act as a control. The stakes are subjectively assessed at regular intervals over a lengthy period of exposure, recommended to be five years or longer (CEN, 1989). The effects of severe microbial biodeterioration on the elasticity and strength properties of wood are used as a guideline, in that a test stake is deemed to have failed if it breaks at the groundline when tapped with a hammer prior to uplifting (CEN, 1989; Borscholt and Henriksen, 1992).

Field testing of a formulation that has already performed well in laboratory tests allows the tester to observe the efficacy of the new preservative by assessing the protective effect in a more representative environment than laboratory testing, and on a larger scale. The guidelines available for field trials have been designed around chemical preservatives, however in this case they have been used as a framework for the design of a field trial assessing a biological control agent. Given the developmental aspect of biological control agents for use in the bioprotection of wood, it is also desirable to include biological tests to provide information about the biocontrol agent, such as its effect on the pattern of colonisation of biocontrol-treated wood or its impact in the surrounding environment. The introduction of biological

testing to this field trial allows monitoring of the biocontrol agent and its effect on the pattern of microbial succession within the substrate (i.e. wooden stakes). Biologically important parameters measured in this field trial included sapstain and soil moisture monitoring; isolation of *Trichoderma* spp. and basidiomycetes; soft rot detection using polarised light microscopy; assessment of wood moisture content; and the measurement of nitrogen content.

Many previously reported field trials assessing biological control agents have tested performance against a limited group of targets. For instance, Bruce and King (1986 a, b; Bruce *et al.*, 1990), described the testing of a *Trichoderma* isolate against *Lentinus* spp. and other selected basidiomycetes in telegraph poles; Score (1998) investigated the biological control of *Serpula lacrymans* using *Trichoderma harzianum*; and Schoeman *et al.* (1995) reported the control of sapstain in freshly felled wood. Examples such as these report the testing of wood bioprotectants against selected target organisms, and have been designed especially for that purpose. This chapter describes the field and fungal cellar testing of a *Trichoderma viride* isolate (T60), applied as a spore suspension to wood stakes. The field trial, based on EN 252 (CEN, 1989), was designed to assess the biocontrol capacity of the isolate under conditions more closely resembling the proposed end-use of ground contact timber treated with a biocontrol agent.

Performance testing of wood preservatives using larger scale wood can take twenty years or more (Johnson *et al.*, 1982), and EN 252 (CEN, 1989) recommends at least 5 years for smaller field trial stakes. In order to obtain information regarding the protective effect of a wood preservative after a relatively short exposure period, an accelerated decay facility may be employed. Accelerated decay facilities are often referred to as fungal cellars and are generally comprised of soil beds containing non-sterile soil, maintained in a controlled environment, with temperature and humidity

elevated to promote rapid decay, although the original "fungal cellar" experiments such as those by Gersonde and other researchers were developed using sterile soil "seeded" with cultures of selected wood decay organisms rather than non-sterile soil (Hainey, 1992) . Due to time constraints, and to supplement the information obtained from the field trial testing of *Trichoderma viride* (T60), a fungal cellar test was also set up in this study, using non-sterile soil.

The field trial described here compares the biocontrol agent to a reference chemical preservative and untreated controls, and employs a range of biological tests to detect and categorise decay, and to assess the effect of the biocontrol agent on the patterns of microbial colonisation and succession. This chapter examines the overall effect on wood decay, i.e. the extent of control, reduction in the rate of decay, type of decay or colonisation by antagonists. The use and adaptation of the standard designed for chemical testing allows a biological control agent to be assessed using the same parameters as a chemical preservative, i.e. to the same point of failure, thereby providing validity for the field trial results when compared with tests carried out for chemical preservatives.

4.2 Materials and methods

Culture maintenance and media preparation were carried out as described in Appendix A: Culture Methods and Isolation. Chemical suppliers are detailed in Appendix B: Chemicals, Reagents and Suppliers. Statistical analyses of selected results are presented in Appendix D.

The treatment of stakes is described in Chapter 3 (3.2.3.2). Test stakes were categorised and labelled according to wood species (spruce or pine), test system (field or cellar) and treatment (CCA; T60; none) as shown in Figure 4.1.

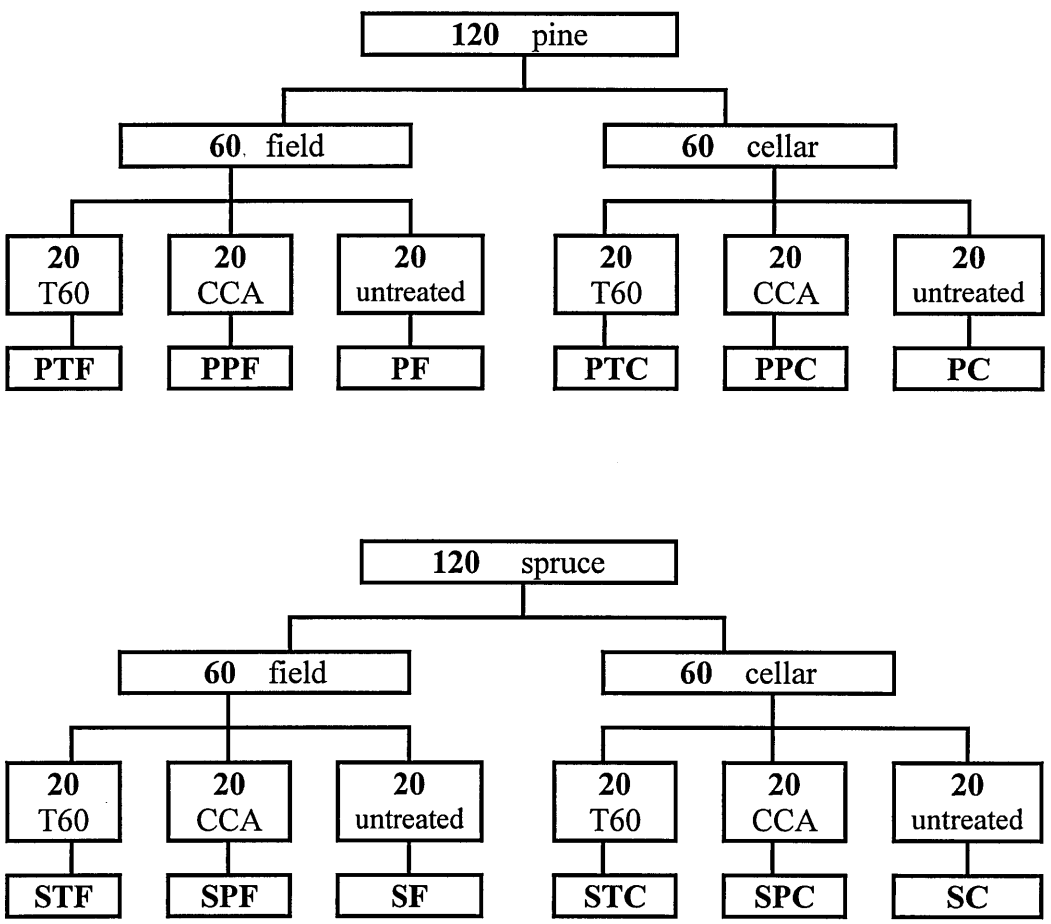


Figure 4.1: Treatment group categories applied to field and fungal cellar test stakes

4.2.1 Placement of stakes at test areas

Field Site

The field site used in this trial is located next to an electricity substation near Tealing, just outside Dundee. Twenty biocontrol-treated stakes of each wood species and 20 preservative-treated stakes of each wood species were buried according to the pattern outlined in EN 252, i.e. to a depth of half their length, at least 30cm apart. Untreated stakes (20 each of pine and spruce) were buried in the same way but in an area slightly removed from the treated stakes to reduce the risk of cross-contamination by the applied biocontrol agent, *Trichoderma viride* T60, as shown in Figure 4.2.

Fungal Cellar

The accelerated decay facility was an irregularly-shaped concrete room of approximately $82m^3$, containing a number of soil beds – large polypropylene tubs measuring 70cm x 100cm x 70cm, each of which contained graded sieved soil prepared as described in Hainey (1992). The soil (agricultural top-soil, sandy loam) was obtained from the Scottish Crop Research Institute (SCRI) in Invergowrie, near Dundee (Hainey, 1992). As with the field site, twenty stakes each of T60-treated, CCA-treated and untreated spruce and pine were buried up to half their length in soil. Ten stakes were planted in each of 12 prepared tubs (see Figure 4.3); again T60-treated and CCA-treated stakes were mixed while untreated stakes were planted in separate tubs. The cellar was maintained at an average temperature of 27°C ($\pm 2^{o}$C). A humidifier was used to maintain the relative humidity of the cellar at as near 90% RH as possible, however in the initial stages of the test, the soil beds were covered in "tents" of clear polythene to compensate for the low relative humidity in the fungal cellar, initially recorded at between 75-80% RH.

Figure 4.2 (a): CCA- and T60-treated stakes in place at the Tealing field site

Figure 4.2 (b): Untreated control stakes, segregated from treated stakes

Figure 4.2: Placement of stakes at the Tealing field site

Figure 4.3: Wooden stakes positioned in a soil bed in the fungal cellar

4.2.2 Monitoring

From this point in the experiment, the methods used were the same for stakes from both the field site and the fungal cellar, with only one exception (virulence controls). The stakes were monitored *in situ* for sapstain discoloration and moisture levels with measurements recorded every 4-6 weeks. Sapstain monitoring was carried out by visually assessing and recording the extent of discoloration for every stake present. As stakes were uplifted for testing during the trial, the numbers of stakes being monitored for sapstain discoloration decreased. A subjective scale of 0-4 was used to represent the degree of discoloration, as described below:

0 – no sapstain visible on wood surfaces

1 – between 0% and 25% coverage of visible wood surfaces

2 – 25 - 50% discoloration

3 – 50 - 75% of visible surface area discoloured

4 – 75 - 100% sapstain discoloration.

Moisture measurements of 5 designated sample stakes from each treatment group (see Figure 4.1) were taken from as close to the groundline as possible and from a point 2 cm below the top of each stake, using an electrical conductivity meter as described in chapter 3 (section 3.2.1). As previously noted, moisture readings recorded in this manner are not accurate, however an indication of the moisture level is obtained.

Soil moisture was monitored with a garden moisture probe, calibrated using soil samples set up at varying moisture contents determined using WHC calculations, as described in Appendix C and Hainey (1992). Measurements were taken from 5

random positions at each of the field site areas (i.e. 5 from within the T60/CCA area and 5 from the untreated control area) and from 2 positions within each soil bed in the fungal cellar.

Virulence controls were used to assess the uniformity of the decay capacity of soil in the fungal cellar. Beech blocks (50 mm longitudinal x 10 mm tangential x 3 mm radial) were buried in each tub for 6 weeks at a time, 6 replicates per tub. Blocks were uplifted, and moisture content and weight loss values were calculated as described in chapter 2 (2.2.1). The assessment was carried out twice, once when test stakes were planted and again shortly after the first uplift.

4.2.3 Preliminary decay assessments

Five replicate stakes of each species from each treatment were selected at random (i.e. 5 CCA-treated spruce stakes, 5 CCA-treated pine stakes etc.) and uplifted after 9 and 18 months exposure. A subjective analysis of stake condition was carried out on site and the wood was then transported to the laboratory for further analysis.

4.2.3.1 Subjective assessment

Using EN 252 as a guideline, stakes were visually examined for obvious signs of decay (i.e. pockets of softened or discoloured wood etc) and other evidence of microbial growth, such as soil adhesion; the presence of hyphal strands; and overall appearance. Surface softness was assessed using a blunted, tapered object, in this case a blunt pencil. A scale of 0-4 was used to represent the soundness of the stake based on the initial examination.

Key: **0** – no evidence of decay or surface softening

1 – slight surface softening, presence of hyphal strands on wood surface

2 – moderate surface softening, presence of decay pockets

3 – severe surface softening, extensive decay pockets

4 – impact failure of stake in field

4.2.3.2 Moisture readings measured by electrical conductivity

Following subjective assessment, moisture contents of the uplifted stakes were measured using a moisture meter (as described in chapter 3, section 3.2.1), at three points along the length of the stake - 4cm from either end of the wood and in the middle of the stake (corresponding with the groundline).

4.2.3.3 Resistance to impact

Measurement of surface softness was also carried out as soon as possible after uplift using a pilodyn, a spring-loaded mechanism (2 Joules; as described by Hainey (1992)) designed to indicate the resistance to impact of wood (Cobra (Wood Treatment) Ltd., Brighton, UK). Readings were taken from both front and back of the stake, at three evenly spaced locations along the length of the wood i.e. top (4cm from the above ground end), middle (at the ground line) and bottom (4cm from the below ground end).

4.2.4 Further decay assessments

4.2.4.1 Basidiomycete isolation

Selective media for isolating basidiomycetes (Clubbe and Levy, 1977) contained benomyl and streptomycin (for the suppression of moulds and bacteria, respectively); media details are presented in Appendix B. Small wood slivers were cut from the edges of any suspect (i.e. discoloured (brown, bleached) or softened) patches present on the surface of the stakes. In addition, 8 evenly spaced slivers of wood were removed from the surface of each stake, around the groundline area (the point to which the stake was buried). This gave a total of 40+ samples per treatment group. The wood samples were surface sterilised by flaming to remove surface contaminants, and placed into the agar. The plates were then incubated at 22°C for 2 weeks and checked daily for signs of basidiomycete growth. Patches of non-basidiomycete growth such as *Mucor* spp. were immediately removed from the plates and any potential basidiomycete growth was sub-cultured to fresh plates. Cultures were confirmed or eliminated as basidiomycetes by observing morphological characteristics such as clamp connections and comparing to photographs in morphological keys (Nobles, 1964; Stalpers, 1978). The isolated cultures were sub-cultured onto gallic acid media (see Appendix B for details), which allowed differentiation of white and brown rot fungi using the Bavendamm test (Nobles, 1948). The test is based on the production of extracellular oxidase enzymes for lignin degradation, which is indicative of a white rot fungal isolate (Carey, 1973).

4.2.4.2 Whole Stake Analysis

Following basidiomycete isolation and prior to further, more destructive analyses, 1 stake from each treatment group was removed for the measurement of whole-stake moisture content and weight loss. Selected stakes were weighed then

oven-dried for 48 hours at 105°C. After cooling, the stakes were re-weighed and the recorded values were used to calculate moisture content and weight loss. Final weights were corrected to account for the uptake of CCA salts or T60 spores during pressure treatment. All subsequent analyses were therefore carried out on the remaining 4 stakes in each treatment group.

4.2.4.3 Isolation of *Trichoderma* spp.

In order to assess the extent of colonisation of the field trial stakes by *Trichoderma* spp., a *Trichoderma* selective media (TSM) developed by Elad *et al.* (1981) was used. The constituents of the selective media are presented in Appendix B. Centimetre-thick cross-sections (1cm longitudinal x 5cm radial x 2.5cm tangential) were cut from the top, groundline ("middle") and bottom of each stake. These sections were then cut into smaller samples numbered 1 to 7, as illustrated in Figure 4.4.

A small wood sliver was removed from the corner of each section and plated out onto TSM. The plates were incubated at 25°C for 2 weeks initially, and examined twice-weekly for the presence or absence of *Trichoderma* spp. Although growth of contaminants did occur this was rare, and easily distinguished from the *Trichoderma* cultures based on their initial morphological appearance.

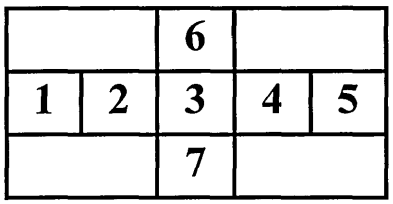


Figure 4.4: Sample positions within a cross-section of a wood stake

4.2.4.4 Determination of Moisture Content

After a small piece of wood was removed from each of the sample blocks (numbered 1-7 for each cross-section) for *Trichoderma* isolation, the blocks were weighed and oven-dried at 105°C overnight. The samples were cooled and re-weighed and the moisture content calculated based on the final dry weight, using the following equation:

$$\text{Moisture Content (\%)} = \frac{\text{final wet weight - final dry weight}}{\text{final dry weight}} \times 100$$

4.2.4.5 Soft Rot Analysis

The remaining sections of the stakes were then assessed for soft rot decay. The three most severe areas of softness on the surface of each stake were noted and samples were taken for analysis using polarised light microscopy. Sections (1mm thick) were removed from each site using a scalpel and placed in a test-tube. Two and a half ml of a 50:50 mixture of glacial acetic acid and 100 volume hydrogen peroxide were added and the tubes placed in a boiling water bath to digest the wood. After 90 minutes the samples were removed, covered and vortexed to completely fiberise the wood. A small amount of the digested wood in solution was placed on a glass slide and examined under polarised light microscopy to detect the presence of the distinctive diamond-shaped cavities that are characteristic of soft rot decay. Ten tracheids were individually assessed for each sample and each tracheid was given a score on a scale of 0-5 according to the degree of soft rot present in the wood cell wall, as described in the following key.

0 - no soft rot cavities

1 - 0 - 24 % of the tracheid wall covered in cavities

2 - 25 % - 49 %

3 - 50 % - 74 %

4 - 75 % - 99 %

5 - complete degradation of the tracheid surface by soft rot cavities

A total soft rot score was then calculated for the 10 tracheids in each sample. The total for each sample was then used to calculate an average score for each treatment group, i.e. 3 soft rot sample locations on each of the 4 remaining stakes giving a mean value of 12 replicates. Sampling continued at increasing alternate depths of 1 mm at each site until little or no soft rot was detected.

4.2.4.6 Determination of Nitrogen Content

Samples (10 mm longitudinal x 50 mm radial x 25 mm tangential) taken from the groundline area of the four stakes remaining in each treatment group were oven-dried, milled and 2 replicate samples of 0.5g each were removed for digestion using the micro-Kjeldahl method (Mowe, 1983). The milled samples were placed in Kjeldahl flasks, and 3ml of concentrated sulphuric acid (18.4M) was carefully added to each flask followed by 2ml of 100 volume hydrogen peroxide. These were heated on a digestion rack for up to 2 hours, adding 1ml of hydrogen peroxide every 30 minutes. Once the samples were completely digested they were allowed to cool before distillation using a Markham apparatus. The nitrogen, released as ammonium gas, was collected (as condensate) in flasks containing 2% boric acid and a few drops of pH indicator (10 parts 0.1% bromocresol green in 95% alcohol + 2 parts 0.1%

methyl red in 95% alcohol). As the solution changed from pink to green, the flasks were removed and back-titrated with 0.01M hydrochloric acid. The amount of nitrogen present in the wood was then expressed as a percentage of the dry mass of the sample using the equation:

$$\text{Nitrogen (\%)} = \frac{\text{titration value} \times 0.14}{\text{weight of sample} \times 10} \times 100$$

In order to determine baseline nitrogen contents for Scots pine and Sitka spruce, 6 blocks of each wood species were taken from untreated, unexposed wood and analysed alongside the field and fungal cellar samples. The mean nitrogen contents were then calculated as described above.

4.3 Results

The following section presents the results of field and fungal cellar testing of the proposed biological control agent *Trichoderma viride* (T60). Test stakes were grouped and labelled according to wood species, treatment and test system; the following figure (Figure 4.5) describes the classification of the treatment groups.

Group	Description	Group	Description
PTF	T60-treated pine, field site	**STF**	T60-treated spruce, field site
PPF	CCA-treated pine, field site	**SPF**	CCA-treated spruce, field site
PF	untreated pine, field site	**SF**	untreated spruce, field site
PTC	T60-treated pine, cellar	**STC**	T60-treated spruce, cellar
PPC	CCA-treated pine, cellar	**SPC**	CCA-treated spruce, cellar
PC	untreated pine, cellar	**SC**	untreated spruce, cellar
1	uplift 1 (9 months)	**2**	uplift 2 (18 months)

Figure 4.5: Key to field trial and fungal cellar treatment groups referred to in this chapter

4.3.1 Monitoring

Sapstain

The results in Table 4.1 show that no sapstain discoloration was observed in any of the CCA-treated stakes, as anticipated. Of the remaining treatment groups, there was less sapstain discoloration in stakes tested in the fungal cellar than in the field stakes, for both pine and spruce. The degree of discoloration was initially higher in the pine stakes than in the spruce, and the onset of sapstain in the untreated pine field samples was recorded earlier than in the T60-treated stakes. This effect was not as pronounced in pine stakes from the fungal cellar. *Trichoderma*-treated spruce stakes initially appeared to discolour at a more rapid rate than untreated spruce stakes in both the field and fungal cellar, however this effect was less evident over time. The field trial control stakes pictured in Figure 4.6 illustrate the increased sapstain discoloration in untreated pine in comparison with untreated spruce.

Figure 4.6: Sapstain discoloration observed in field trial stakes. Note the increased discoloration in untreated pine (top-centre of photograph) in comparison with untreated spruce (immediately left and right of discoloured pine stakes).

Time	Number	PTF	PPF	PF	PTC	PPC	PC
10 weeks	20	0.9 (0.5)	0.0 (0.0)	2.2 (0.6)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
12 weeks	20	1.7 (0.7)	0.0 (0.0)	2.7 (0.5)	0.0 (0.0)	0.0 (0.0)	0.1 (0.2)
18 weeks	20	2.3 (0.9)	0.0 (0.0)	3.1 (0.8)	0.2 (0.4)	0.0 (0.0)	0.2 (0.4)
22 weeks	20	2.7 (0.7)	0.0 (0.0)	3.5 (0.5)	0.4 (0.5)	0.0 (0.0)	0.4 (0.5)
28 weeks	20	3.6 (0.6)	0.0 (0.0)	3.7 (0.5)	0.7 (0.5)	0.0 (0.0)	0.9 (0.6)
36 weeks	20	3.7 (0.5)	0.0 (0.0)	3.8 (0.4)	0.9 (0.5)	0.0 (0.0)	1.1 (0.6)
50 weeks	15	3.9 (0.3)	0.0 (0.0)	3.9 (0.2)	1.6 (0.5)	0.0 (0.0)	1.8 (0.6)
54 weeks	15	4.0 (0.0)	0.0 (0.0)	4.0 (0.0)	1.9 (0.7)	0.0 (0.0)	2.1 (0.6)
64 weeks	15	4.0 (0.0)	0.0 (0.0)	4.0 (0.0)	2.7 (0.8)	0.0 (0.0)	2.7 (0.6)
70 weeks	15	4.0 (0.0)	0.0 (0.0)	4.0 (0.0)	3.1 (1.0)	0.0 (0.0)	3.1 (0.6)

Table 4.1 (a): Pine stakes

Time	Number	STF	SPF	SF	STC	SPC	SC
10 weeks	20	0.9 (0.5)	0.0 (0.0)	0.2 (0.4)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
12 weeks	20	1.3 (0.4)	0.0 (0.0)	0.7 (0.5)	0.4 (0.5)	0.0 (0.0)	0.1 (0.2)
18 weeks	20	1.7 (0.5)	0.0 (0.0)	0.8 (0.6)	0.4 (0.5)	0.0 (0.0)	0.2 (0.4)
22 weeks	20	2.3 (0.8)	0.0 (0.0)	1.7 (0.6)	0.6 (0.5)	0.0 (0.0)	0.4 (0.5)
28 weeks	20	2.7 (0.7)	0.0 (0.0)	2.2 (0.5)	1.8 (0.5)	0.0 (0.0)	0.7 (0.5)
36 weeks	20	3.8 (0.4)	0.0 (0.0)	3.2 (0.6)	1.9 (0.6)	0.0 (0.0)	0.9 (0.5)
50 weeks	15	3.9 (0.3)	0.0 (0.0)	3.7 (0.4)	2.3 (0.8)	0.0 (0.0)	1.3 (0.5)
54 weeks	15	4.0 (0.0)	0.0 (0.0)	3.9 (0.3)	2.4 (0.8)	0.0 (0.0)	1.8 (0.4)
64 weeks	15	4.0 (0.0)	0.0 (0.0)	4.0 (0.0)	2.6 (0.7)	0.0 (0.0)	2.3 (0.7)
70 weeks	15	4.0 (0.0)	0.0 (0.0)	4.0 (0.0)	2.7 (0.7)	0.0 (0.0)	2.7 (0.7)

Table 4.1 (b): Spruce stakes

Table 4.1: Mean visual assessment scores for sapstain discoloration observed in field and fungal cellar stakes. Figures in parentheses represent standard deviations.

"**Number**" (column 2) refers to the total number of stakes assessed per treatment group; the numbers assessed decreased from 20 to 15 following uplift 1 when 5 stakes from each treatment category were removed for analysis.

Key: 0 - no sapstain discoloration; **1** - <25% discoloration; **2** - 25-50% discoloration; **3** - 50-75% discoloration; **4** - >100% discoloration.

Wood and soil moisture

Soil moisture measurements were recorded during the field tests using a garden moisture probe. Readings from the probe were converted into moisture contents (%) using the calibration method shown in Appendix C; and soil moisture contents measured over time are displayed in Figure 4.7. The results show moisture levels to be lower in the field site soil than in the fungal cellar throughout the testing period, with fluctuations in moisture readings indicative of seasonal variations. Within 3 months of commencing accelerated decay testing, fungal cellar soil moisture levels were extremely high i.e. above 30% (recorded as "10+" on the soil moisture meter). In addition to this, a degree of variation was observed between soil beds, particularly at the start of the fungal cellar testing period.

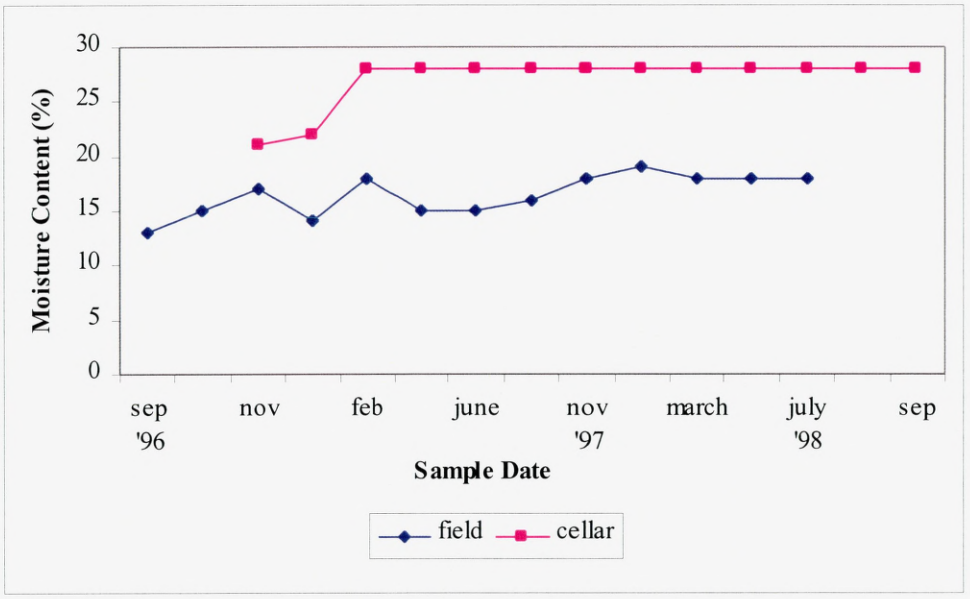


Figure 4.7: Soil moisture content (%) recorded during field and fungal cellar testing

Monitoring of wood moisture levels in field and fungal cellar stakes (using a moisture meter) is represented in Figure 4.8 (a-f). The charts indicate that the difference between wood moisture at the groundline and at the top of the stakes was more pronounced in the field stakes than in the cellar stakes, due to the environmental differences between the field and cellar. There appears to have been less variation in the moisture levels over time in CCA-treated monitoring stakes than in non-CCA treated wood, and CCA-treated wood was consistently found to have lower moisture levels than other treatments. The charts also show higher variability of moisture levels recorded in T60-treated wood over time compared with untreated wood and in general, lower moisture readings were observed in untreated field stakes and CCA-treated cellar stakes than in T60-treated wood.

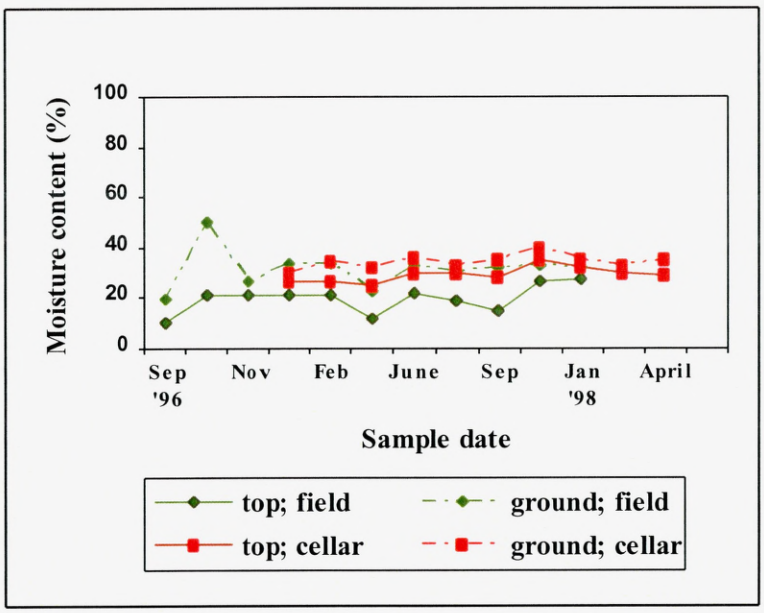


Figure 4.8 (a): Moisture content monitored over time in CCA-treated pine stakes

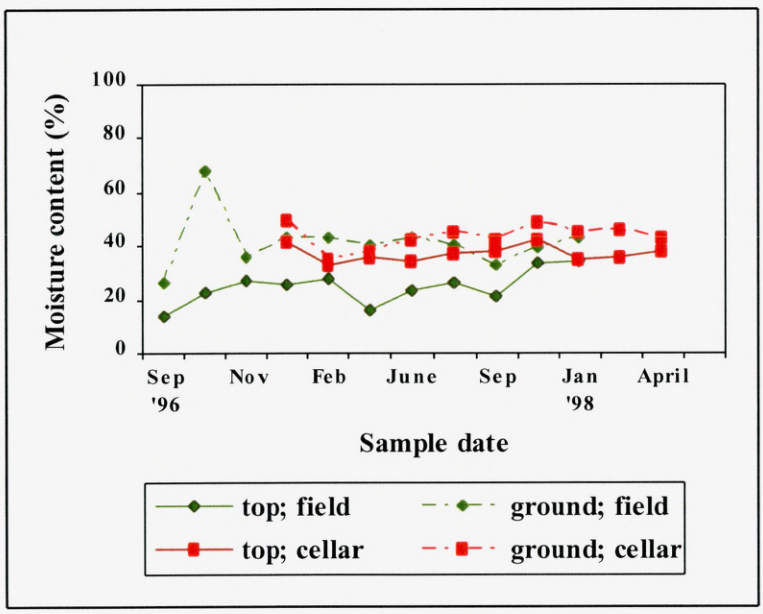


Figure 4.8 (b): Moisture contents recorded over time, in CCA-treated spruce stakes

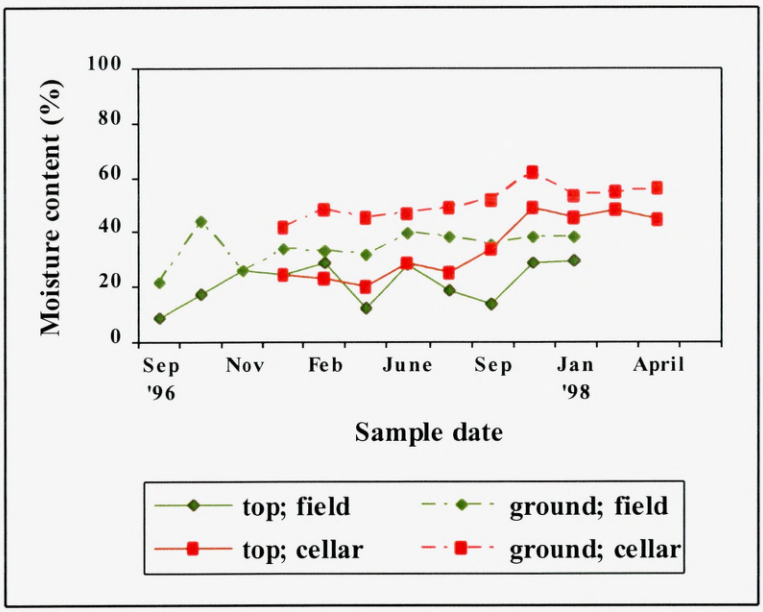


Figure 4.8 (c): Moisture content recorded over time in T60-treated pine stakes

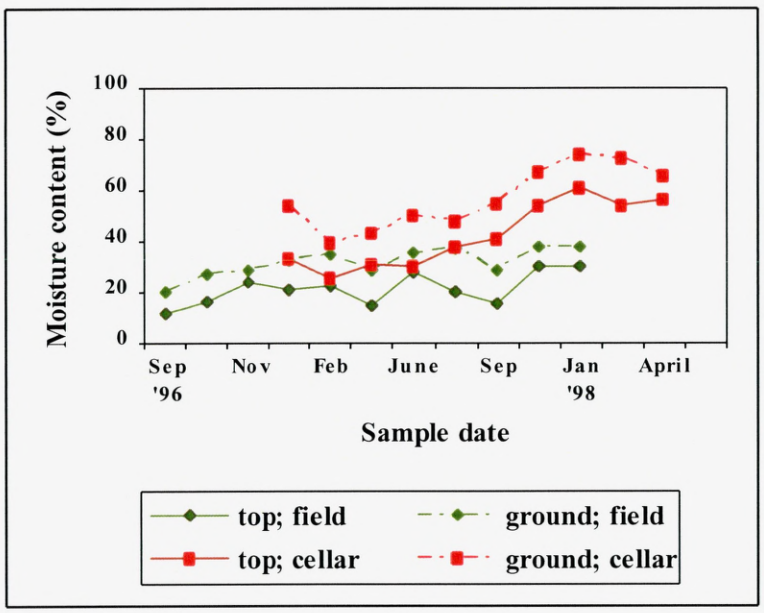


Figure 4.8 (d): Moisture content recorded over time in T60-treated spruce stakes

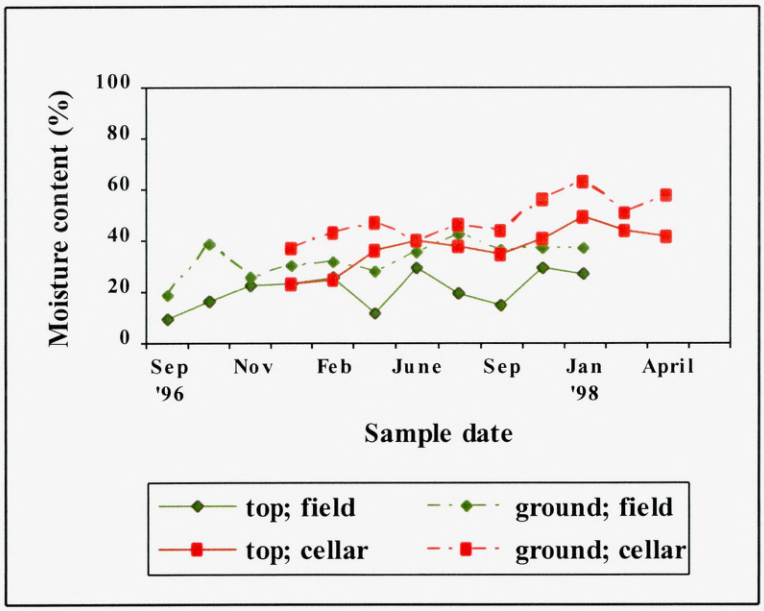


Figure 4.8 (e): Moisture content recorded over time in untreated pine controls

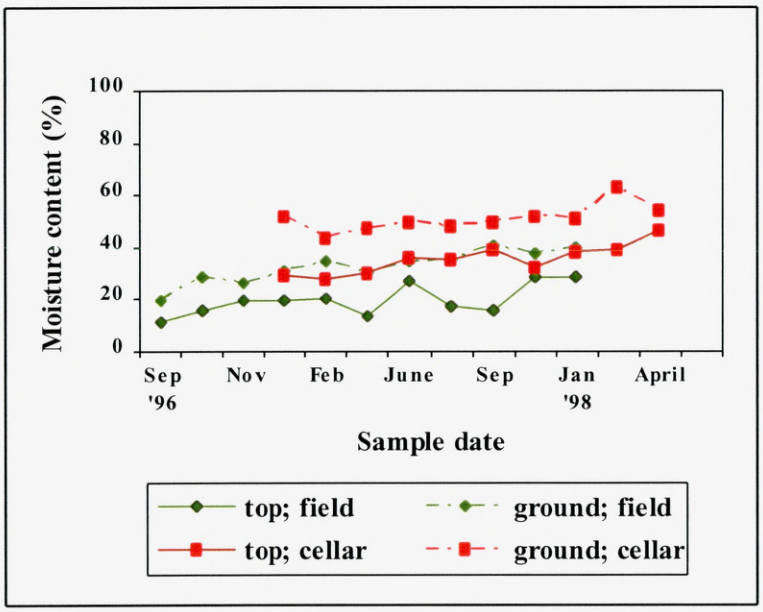


Figure 4.8 (f): Moisture content recorded over time in untreated spruce controls

Figure 4.8: Wood moisture content monitoring of field and fungal cellar stakes. Moisture contents were measured near the top of the stake ("top") and at the groundline ("ground") of 5 stakes per treatment group.

Table 4.2 and Figure 4.9 show the moisture contents and weight losses of virulence controls used to assess the decay capacity of the soil in the fungal cellar. The tub-to-tub variations in moisture content noted during soil moisture monitoring are reflected in these results, as soil moisture content will have a direct effect on wood moisture content. Figure 4.9 shows that the variation in moisture content of wood blocks recovered from each soil bed was more pronounced at the start of the trial (uplift 1) than in later samples. The results from the second uplift of virulence controls indicated that soil moisture variations had been resolved and wood block moisture contents did not vary as much with regard to tub number, although moisture contents from the second virulence test were higher than those recorded for the first set of test blocks. An acceptable level of decay was achieved in virulence controls from both test periods, indicating that fungal cellar soil was appropriate for accelerated decay testing. Although recorded weight losses were slightly lower in blocks from uplift 2, Figure 4.9 (a) demonstrates that a similar tub-to-tub weight loss pattern can be seen in uplift 2 as in uplift 1. Wood moisture contents recorded after uplift 2 of the virulence control blocks were higher than moisture contents from uplift 1, however less tub-to-tub variation in moisture contents were observed in uplift 2 blocks. Although wood moisture content can influence fungal colonisation and decay, the absence of a similar pattern of tub-to-tub variation in uplift 2 moisture contents to that observed in uplift 2 weight losses indicated that the reduced level of decay was not solely the result of elevated wood moisture contents.

Uplift 1		Tub N°.	Uplift 2	
Weight loss (%)	Moisture content (%)		Weight loss (%)	Moisture content (%)
22.6 (4.2)	77.4 (7.9)	1	10.6 (3.7)	140.7 (6.2)
24.2 (6.1)	91.6 (2.7)	2	10.3 (0.7)	143.2 (4.9)
22.6 (4.9)	140.5 (15.6)	3	19.3 (4.5)	160.3 (9.8)
30.1 (2.3)	59.8 (10.1)	4	30.9 (2.7)	166.6 (3.0)
31.2 (6.7)	73.2 (14.4)	5	16.9 (5.1)	152.8 (6.2)
30.7 (2.2)	142.5 (17.4)	6	22.7 (3.6)	165.2 (9.2)
21.2 (2.1)	133.3 (8.3)	7	19.0 (3.5)	161.9 (14.1)
32.8 (2.3)	52.7 (5.6)	8	25.2 (1.4)	135.5 (15.9)
25.0 (3.6)	47.6 (8.1)	9	27.9 (2.2)	157.8 (23.2)
33.2 (2.2)	70.0 (6.7)	10	25.5 (1.5)	161.2 (8.6)
30.2 (5.4)	48.4 (2.9)	11	21.9 (2.3)	163.5 (9.5)
35.1 (2.9)	111.0 (9.3)	12	21.9 (2.6)	174.7 (5.1)

Table 4.2: Mean weight losses (%) and moisture contents (%) of beech blocks used as virulence controls in the fungal cellar. Means are for a total of 6 replicates per tub at each uplift. Figures in parentheses represent standard deviations.

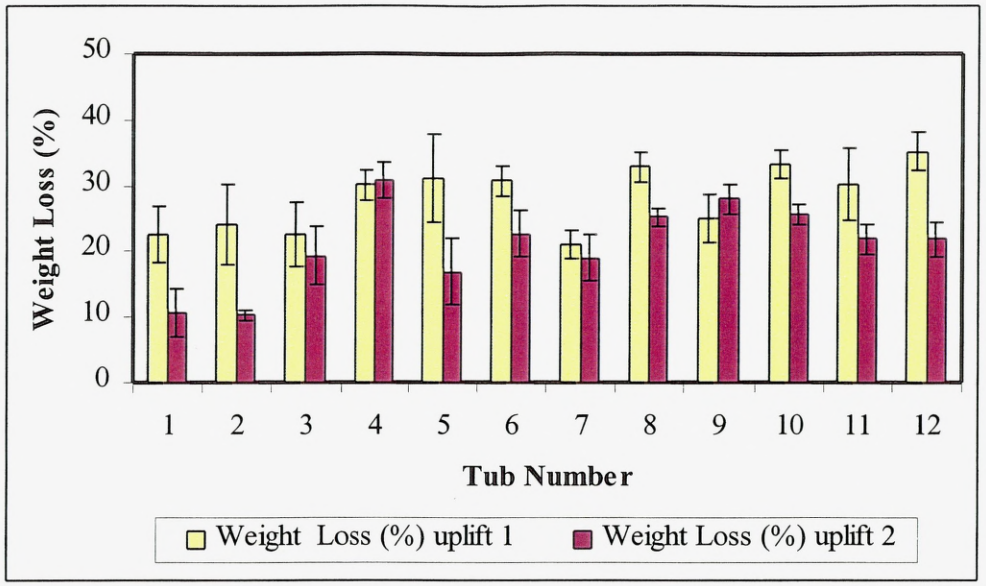


Figure 4.9 (a): Weight losses (%) of virulence controls in the fungal cellar.

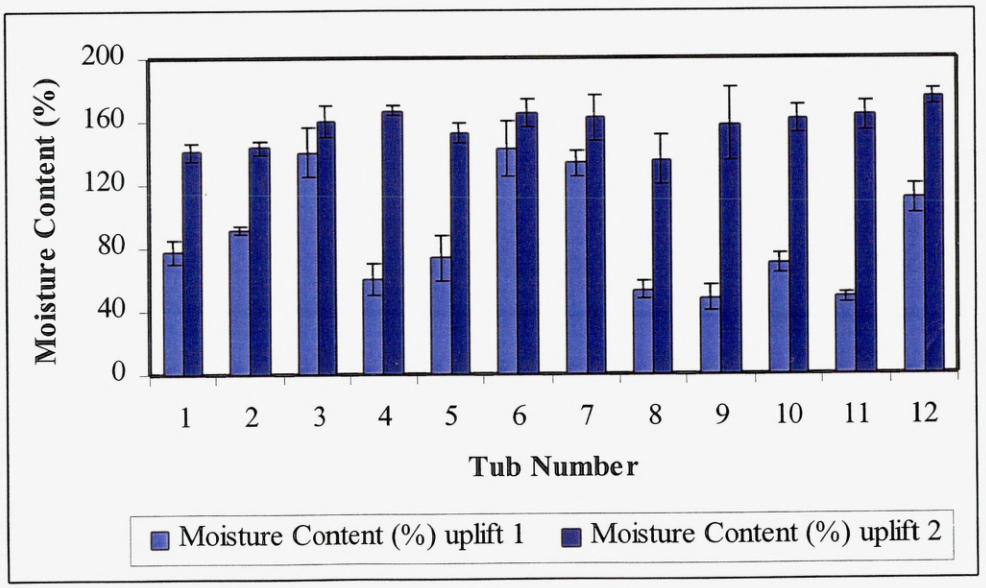


Figure 4.9 (b): Moisture contents (%) of virulence controls in the fungal cellar.

Figure 4.9: Weight losses (%) and moisture contents (%) of virulence controls in the fungal cellar.

4.3.2 Preliminary decay assessments

4.3.2.1 Subjective assessment

Very little decay was observed during subjective assessment of stakes uplifted from the field after 9 months (uplift 1), as indicated by the results in Table 4.3, but there was sufficient alteration in appearance after 18 months (uplift 2) to show a slight reduction in biodegradation within the T60-treated stakes. Figure 4.10 shows a sound T60-treated pine stake being subjectively assessed. Impact failure was not observed in any of the CCA- or T60-treated stakes in either the field or fungal cellar. Three untreated cellar stakes however, (2 spruce stakes and 1 pine stake) failed when they broke at the groundline whilst being removed from the soil beds after 18 months. Figure 4.11 shows one of the failed spruce stakes photographed at the point of failure; the uneven break point and discoloured internal wood indicate substantial brown rot degradation has occurred. Most of the CCA-treated stakes showed no signs of wood degrade, although some CCA-treated spruce stakes uplifted after 18 months in the fungal cellar displayed a small amount of patchy softening below the groundline. However, no further evidence of decay was observed in preservative treated stakes and the results of moisture content measurements (taken from corresponding positions) indicate that softening could have been a result of localised moisture increases. Although no statistically significant protective effects were noted in spruce field stakes or in fungal cellar stakes of either wood species, there was a significant reduction (at $p \geq 0.05$) in the subjective assessment scores of T60-treated pine field stakes compared to corresponding pine controls (see Appendix D (iii), Table 4.3).

Uplift 1		
PTF	**PPF**	**PF**
0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

Uplift 2		
PTF	**PPF**	**PF**
1.4 (0.4)	0 (0.0)	2.4 (0.5)

Table 4.3 (a): Pine stakes from field tests

Uplift 1		
PTC	**PPC**	**PC**
1.0 (0.5)	0.0 (0.0)	1.4 (0.4)

Uplift 2		
PTC	**PPC**	**PC**
2.5 (0.5)	0.0 (0.0)	2.8 (0.8)

Table 4.3 (b): Pine stakes from cellar tests

Uplift 1		
STF	**SPF**	**SF**
0.0 (0.0)	0.0 (0.0)	0.2 (0.4)

Uplift 2		
STF	**SPF**	**SF**
1.2 (0.3)	0.0 (0.0)	1.8 (0.3)

Table 4.3 (c): Spruce stakes from field tests

Uplift 1		
STC	**SPC**	**SC**
0.8 (0.4)	0.0 (0.0)	1.2 (0.6)

Uplift 2		
STC	**SPC**	**SC**
3.0 (0.0)	0.4 (0.5)	3.2 (0.8)

Table 4.3 (d): Spruce stakes from cellar tests

Table 4.3: Mean scores from subjective assessment of field and fungal cellar stakes. Five stakes were examined per treatment group at each uplift, immediately following removal from the soil. Figures in parentheses represent standard deviations. Statistical analyses (ANOVA) are presented in Appendix D (i -iii).

Key: 0 – no evidence of decay or surface softening

1 – slight surface softening, presence of hyphal strands on wood surface

2 – moderate surface softening, presence of decay pockets

3 – severe surface softening, extensive decay pockets

4 – impact failure of stake in position

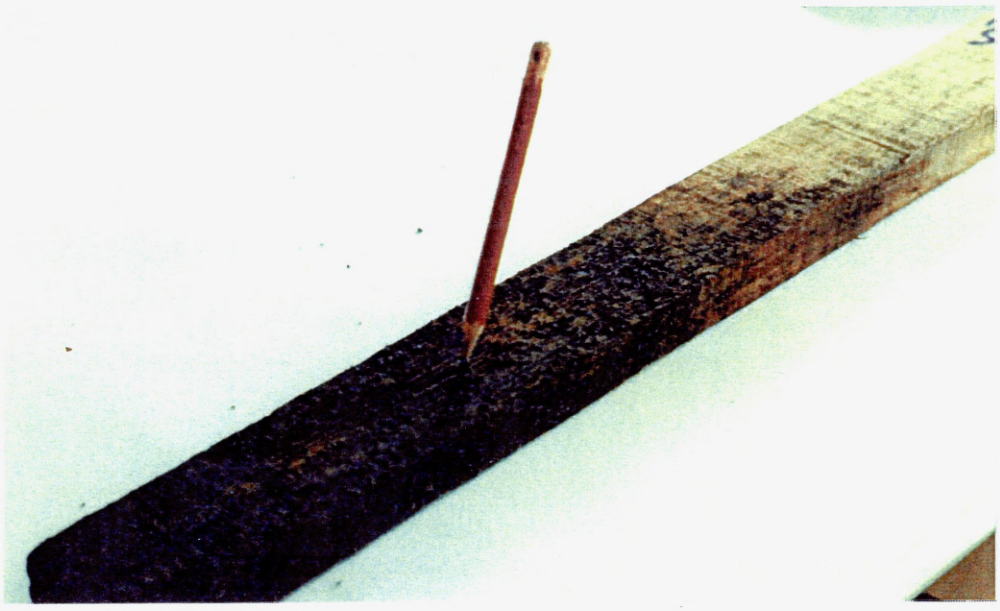


Figure 4.10: Subjective assessment of a *Trichoderma*-treated pine stake.

Figure 4.11: Impact failure of an untreated fungal cellar spruce stake.

4.3.2.2 Moisture readings measured by electrical conductivity

The moisture of wood uplifted for assessment was measured using electrical conductivity (i.e. a moisture meter) for a preliminary indication of moisture levels at the stake surface (Table 4.4 (a-d)). Stakes uplifted after 9 months in the field showed a marked increase in moisture readings at groundline and below ground sample points than above ground, as expected (Table 4.4 (a) and (c)). The moisture level results also show that after 9 months in the field, wood above ground level was not at fibre saturation point. Although there were few significant differences between treatment groups in pine, spruce stakes uplifted from the field after 9 months displayed statistically significant differences between T60-treated and CCA-treated spruce, with the preservative treated wood showing distinctly higher moisture levels (see Appendix D (iii), Table 4.4). After 18 months in the field, stakes from all treatment groups displayed significantly higher moisture readings in above ground sample points than at the earlier uplift, and a similar increase was also apparent at most groundline and below groundline sample points. In general, there were few statistically significant differences observed between the moisture levels of T60-treated field stakes and those of untreated control stakes.

Samples uplifted after 9 months exposure to soil in the fungal cellar showed a significant increase in moisture of T60-treated pine above and below the groundline in comparison to untreated pine, while the moisture readings recorded at the groundline sampling points were similar for both of these treatment groups (Table 4.4 (b)). A similar pattern was seen in T60-treated spruce (Table 4.4 (d)); however the standard deviation value for the above groundline sample point means that the difference from untreated spruce is not significant (see Appendix D (iii), Table 4.4). All moisture levels recorded in fungal cellar stakes after 9 months were significantly higher than those of the corresponding field samples (see Appendix D (ii), Table 4.4).

After 18 months in the fungal cellar, T60-treated stakes did not display significantly higher moisture levels than untreated controls and in general, moisture readings for T60-treated stakes were not significantly higher after 18 months cellar exposure than those from uplift 1 (see Appendix D (i), Table 4.4).

Uplift 1				Uplift 2		
PTF	PPF	PF		PTF	PPF	PF
12.8 (1.3)	13.7 (0.4)	11.3 (0.4)	top	24.5 (5.0)	24.4 (0.7)	27.8 (5.3)
28.8 (1.4)	29.1 (1.1)	29.3 (1.2)	middle	38.0 (2.3)	32.8 (1.7)	38.7 (5.9)
29.0 (0.2)	27.9 (1.4)	28.8 (1.7)	bottom	34.12 (1.1)	31.1 (2.2)	36.9 (2.5)

Table 4.4 (a): Pine stakes from field tests

Uplift 1				Uplift 2		
PTC	PPC	PC		PTC	PPC	PC
26.0 (4.2)	24.4 (2.8)	18.6 (1.1)	top	36.7 (10.5)	23.7 (1.2)	21.6 (3.7)
56.2 (3.6)	35.1 (3.6)	57.5 (2.1)	middle	58.9 (5.0)	37.2 (1.6)	66.5 (4.7)
50.0 (1.9)	40.3 (5.7)	39.2 (5.4)	bottom	55.0 (5.5)	38.2 (2.8)	53.2 (6.5)

Table 4.4 (b): Pine stakes from cellar tests

Uplift 1				Uplift 2		
STF	SPF	SF		STF	SPF	SF
13.2 (0.9)	16.0 (0.3)	13.8 (0.1)	top	19.7 (2.1)	31.8 (2.0)	23.5 (4.0)
29.9 (2.0)	35.6 (2.8)	29.5 (2.4)	middle	38.3 (2.0)	40.1 (4.2)	38.6 (4.8)
32.8 (2.0)	37.8 (1.7)	30.0 (2.4)	bottom	38.2 (1.4)	38.2 (1.6)	32.8 (0.9)

Table 4.4 (c): Spruce stakes from field tests

Uplift 1				Uplift 2		
STC	SPC	SC		STC	SPC	SC
41.8 (19.5)	33.4 (2.3)	24.0 (2.5)	top	34.8 (19.5)	29.8 (1.7)	21.5 (1.2)
65.4 (8.1)	43.4 (4.0)	54.3 (7.1)	middle	67.9 (5.1)	47.2 (4.9)	73.2 (6.8)
58.1 (4.8)	50.6 (4.6)	43.3 (2.1)	bottom	63.8 (2.2)	60.4 (5.5)	67.0 (2.4)

Table 4.4 (d): Spruce stakes from cellar tests

Table 4.4: Mean moisture levels of field and fungal cellar stakes measured using a moisture meter. Measurements were taken immediately following uplift. Values are means of 5 measurements (1 per position per stake per treatment group). Figures in parentheses represent standard deviations. Results of ANOVA analysis are presented in Appendix D (i-iii).

4.3.2.3 Resistance to impact

Wood uplifted after 9 months in the field was tested for surface softness using a pilodyn (Table 4.5, parts (a) and (c)). The results reflected a similar pattern to the electrical conductivity measurement of moisture content, in that the tops of the stakes were harder than middle or below ground points. Statistically significant differences were observed in pilodyn readings between CCA-treated pine and T60-treated pine at all sample points, and between CCA-treated pine and untreated pine at the groundline, however there were no statistical differences between the different treatment groups in spruce (Appendix D (iii), Table 4.5). The results in Table 4.5 (a) and (c) also indicate that the spruce stakes were softer than pine, most likely due to the different densities of the wood species. After 18 months in the field site, there was a significant decrease in resistance to impact in above ground sampling points in T60- and untreated wood of both species, but only a moderate increase in softening was observed at points in ground contact over time. CCA-treated spruce uplifted after 18 months in the field was significantly harder than either T60-treated or untreated spruce. ANOVA analysis also indicated that T60-treated spruce uplifted after 18 months was significantly more resistant to impact below ground than the corresponding untreated controls at the same position.

Stakes uplifted after 9 months in the fungal cellar (Table 4.5 (b) and (d)) showed a similar gradient of resistance to impact as field stakes, but with a slightly higher rate of variability between individual spruce stakes. There were no significant differences in resistance to impact between T60-treated and untreated wood, and the CCA-treated stakes again demonstrated a higher resistance to impact than non-CCA treated wood. The second uplift of stakes from the fungal cellar did not indicate any significant increases in surface softness in pine stakes compared with uplift 1, and the results in Table 4.5 (b) indicate increased resistance to impact in T60-treated pine

compared with untreated pine at the groundline and below, however these differences were not found to be statistically significant (Appendix D (iii), Table 4.5). Spruce stakes uplifted after 18 months in the cellar showed T60-treated wood in ground contact to have a reduced surface softness in comparison to untreated spruce. As was expected, very little significant reduction was recorded in the resistance to impact of CCA stakes over the 18 month test period, in either field or fungal cellar samples.

Uplift 1				Uplift 2		
PTF	PPF	PF		PTF	PPF	PF
6.5 (0.8)	5.6 (0.6)	6.1 (0.9)	top	8.5 (1.4)	6.7 (1.4)	8.2 (0.8)
10.0 (1.2)	7.8 (1.3)	9.6 (1.0)	middle	10.0 (2.3)	8.8 (1.1)	11.1 (3.4)
10.7 (1.4)	9.1 (1.0)	9.7 (1.1)	bottom	9.4 (1.0)	8.8 (1.1)	10.0 (1.3)

Table 4.5 (a): Pine stakes from field tests

Uplift 1				Uplift 2		
PTC	PPC	PC		PTC	PPC	PC
6.8 (1.3)	4.8 (0.8)	6.5 (0.6)	top	8.7 (3.1)	5.0 (0.6)	5.3 (1.2)
11.1 (2.1)	6.6 (1.2)	13.0 (4.7)	middle	12.2 (2.8)	6.9 (0.7)	16.4 (6.8)
11.6 (1.8)	7.4 (1.4)	12.0 (2.4)	bottom	11.9 (2.6)	7.5 (1.1)	13.9 (2.8)

Table 4.5 (b): Pine stakes from cellar tests

Uplift 1				Uplift 2		
STF	SPF	SF		STF	SPF	SF
9.4 (1.5)	9.4 (2.1)	8.1 (1.4)	top	12.2 (1.2)	10.1 (2.9)	11.4 (2.3)
15.3 (2.6)	13.4 (1.6)	13.7 (3.5)	middle	16.7 (1.5)	13.4 (1.7)	16.1 (5.1)
16.2 (3.2)	13.9 (2.0)	13.4 (3.0)	bottom	11.8 (2.0)	12.4 (2.6)	14.8 (2.5)

Table 4.5 (c): Spruce stakes from field tests

Uplift 1				Uplift 2		
STC	SPC	SC		STC	SPC	SC
11.4 (2.1)	8.5 (1.4)	10.2 (2.0)	top	10.9 (2.2)	8.6 (1.6)	10.8 (1.2)
14.9 (1.9)	12.7 (1.9)	15.2 (5.8)	middle	17.9 (3.6)	11.2 (2.7)	24.2 (4.1)
15.2 (2.7)	13.1 (1.7)	15.7 (5.1)	bottom	14.4 (1.0)	12.8 (2.5)	19.1 (4.5)

Table 4.5 (d): Spruce stakes from cellar tests

Table 4.5: Resistance to impact measured using a pilodyn. Depth of pin penetration measured in millimetres (mm). Values represent the mean of 10 readings. Figures in parentheses represent standard deviations. Statistical analyses are presented in Appendix D (i-iii).

4.3.3 Further decay assessments

4.3.3.1 Basidiomycete isolation

The results in Table 4.6 (a-d) show the frequency of basidiomycetes isolated from field and fungal cellar stakes, per treatment group (i.e. total number of cultures obtained from 40+ samples). The overall rate of basidiomycete isolation was low, and the abundant growth of contaminants despite the use of inhibitors, further hampered isolation. Fewer basidiomycetes were isolated from T60-treated pine field trial stakes in comparison to untreated pine (Table 4.6 (a)), and there was also a lower frequency of isolation from T60-treated spruce from the cellar in relation to untreated spruce (Table 4.6 (d)). One sample out of more than 40 wood slivers removed from the surface of CCA-treated pine stakes, uplifted from the cellar after 18 months, resulted in basidiomycete growth, perhaps from a surface contaminant; however, there were no indications of decay in CCA-treated pine during subjective assessment. Potential basidiomycete growth was excised and sub-cultured as described in 4.2.4.1, and cultures were identified as basidiomycetes primarily on the basis of clamp connections observed under microscopic examination.. Specific identification of basidiomycetes was not undertaken, however results of tannic acid sub-culturing indicated that all fungi isolated as basidiomycetes were brown rot fungi.

Uplift 1		
PTF	PPF	PF
0	0	0

Uplift 2		
PTF	PPF	PF
1	0	2

Table 4.6 (a): Pine stakes from field tests

Uplift 1		
PTC	PPC	PC
0	0	1

Uplift 2		
PTC	PPC	PC
2	1	4

Table 4.6 (b): Pine stakes from cellar tests

Uplift 1		
STF	SPF	SF
0	0	1

Uplift 2		
STF	SPF	SF
1	0	2

Table 4.6 (c): Spruce stakes from field tests

Uplift 1		
STC	SPC	SC
0	0	1

Uplift 2		
STC	SPC	SC
2	0	5

Table 4.6 (d): Spruce stakes from cellar tests

Table 4.6: Frequency of basidiomycete isolation from field and fungal cellar stakes. Values are the total number of clean cultures recovered from 40+ samples per treatment group.

4.3.3.2 Whole stake analysis

Table 4.7 presents the weight loss and moisture content results obtained from whole stake analysis of 1 stake from each treatment group. Very little overall weight loss was observed in field samples, even after 18 months, which makes it difficult to determine the presence or absence of a protective effect. With the exception of pine stakes uplifted after 9 months in the field, higher moisture contents were observed in all T60-treated stakes in comparison to untreated controls. Spruce cellar stakes demonstrated a higher moisture content and weight loss than their pine counterparts, but with only 1 stake analysed per treatment group there is no means of determining statistical significance.

After 9 months in the fungal cellar, the T60-treated pine stake showed less weight loss than the untreated pine control, although there was more weight loss in the T60-treated stake than in the CCA-treated pine. There were also clear differences in moisture content, with T60-treated pine demonstrating a higher moisture content than either CCA-treated or untreated pine. The CCA-treated pine stake had a particularly low moisture content for a fungal cellar specimen, probably due to the effects of the chemical preservative (Jonnsson *et al.*, 1989). The same pattern in moisture content was evident in stakes uplifted after 18 months in the fungal cellar, however there was no longer any indication of decay reduction in T60-treated pine. The *Trichoderma*-treated spruce stake uplifted after 9 months demonstrated a marked reduction in weight loss in comparison to untreated spruce, and although this effect was not as pronounced after 18 months, there is still a clear reduction in weight loss in T60-treated spruce with regard to untreated controls. The moisture contents of T60-treated wood were again much higher than those of untreated or CCA-treated wood.

Uplift 1				Uplift 2		
PTF	**PPF**	**PF**		**PTF**	**PPF**	**PF**
3.52	1.48	2.07	**Wt. Loss (%)**	3.71	1.05	4.62
42.80	29.32	48.23	**MC (%)**	88.68	66.18	62.36

Table 4.7 (a): Pine stakes from field tests

Uplift 1				Uplift 2		
PTC	**PPC**	**PC**		**PTC**	**PPC**	**PC**
9.74	2.56	11.74	**Wt. Loss (%)**	14.22	2.79	13.52
95.32	39.89	61.51	**MC (%)**	119.58	49.88	80.63

Table 4.7 (b): Pine stakes from cellar tests

Uplift 1				Uplift 2		
STF	**SPF**	**SF**		**STF**	**SPF**	**SF**
1.02	0.97	0.90	**Wt. Loss (%)**	2.77	1.24	4.22
78.34	65.40	41.59	**MC (%)**	137.73	107.15	71.78

Table 4.7 (c): Spruce stakes from field tests

Uplift 1				Uplift 2		
STC	**SPC**	**SC**		**STC**	**SPC**	**SC**
6.45	1.70	21.18	**Wt. Loss (%)**	21.94	3.06	29.87
130.76	80.11	62.14	**MC (%)**	138.52	97.51	91.11

Table 4.7 (d): Spruce stakes from cellar tests

Table 4.7: Weight loss (%) and moisture content (%) of whole stakes after exposure to ground contact (1 stake per uplift group).

4.3.3.3 Isolation of *Trichoderma* spp.

The results of the selective media isolation of *Trichoderma* spp. from field and fungal cellar stakes are presented in Figure 4.12. In general, these results indicate better colonisation in pine stakes than in spruce stakes, and most groundline areas showed a higher frequency of isolation than above ground samples. The observed frequency of isolation of *Trichoderma* spp. from T60-treated field stakes of both wood species was lower after 18 months than after 9 months in field samples, indicating a potential problem with maintaining a consistent presence of the biocontrol agent. This may be due to either seasonal variation in growing conditions, since similar patterns were not seen in fungal cellar stakes; death (or dormancy) of the applied biocontrol agent under field conditions; or loss of available nutrients over time. However, isolation frequencies for untreated and CCA-treated stakes of both species also demonstrated this pattern, suggesting a seasonal influence on antagonist growth and population in field conditions. There was no observed effect of pre-treatment with *Trichoderma* spores on the frequency of isolation, with little difference between T60-treated and untreated control stakes. Untreated control stakes were segregated from T60-treated stakes, particularly at the field site, which suggests that the control stakes were exhibiting natural colonisation by *Trichoderma* spp. present in the soil. As anticipated, there was a lower frequency of isolation from CCA-treated stakes than non-CCA-treated stakes although *Trichoderma* was still isolated from preservative-treated samples.

Stakes uplifted from the fungal cellar demonstrated more consistent isolation from stakes across the two test periods (i.e. frequency of isolation after 9 months is similar to that after 18 months). This was observed for all three treatment groups (T60; CCA; untreated), and both wood species. There was a slightly higher frequency of isolation from untreated cellar controls than T60-treated controls, suggesting that

natural levels of *Trichoderma* present in the fungal cellar soil are high. Overall, there was a higher frequency of *Trichoderma* isolation from pine stakes than spruce (potentially due to the higher nutrient content of pine), and a lower frequency of isolation was observed in stakes from most CCA treatment groups.

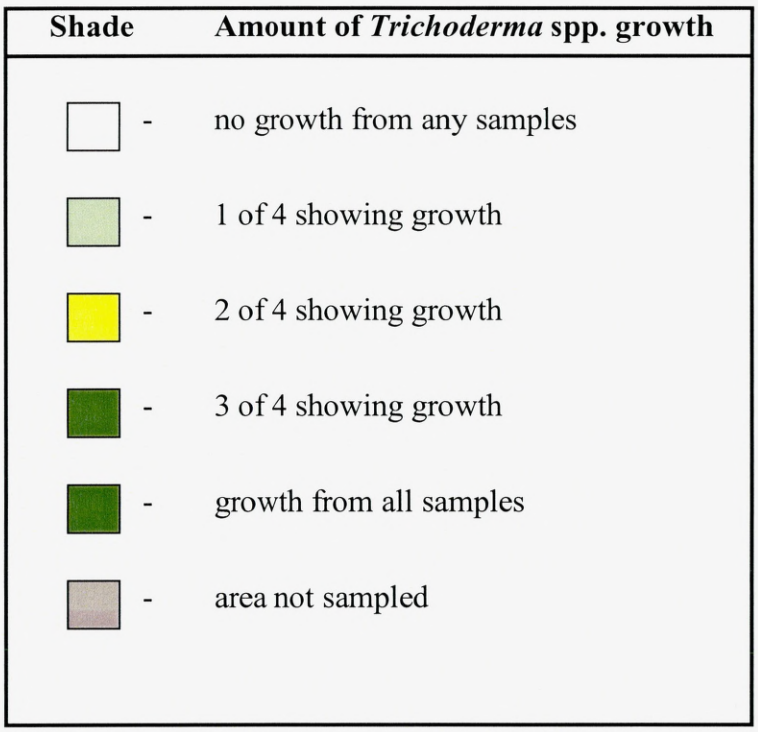


Figure 4.12 (a): Key to Figure 4.12 parts (b) and (c), where shading of cross-section templates represents the amount of growth recorded. Treatment group codes are the same as described in Figure 4.5.

Figure 4.12: Frequency of *Trichoderma* spp. isolation from field trial and fungal cellar stakes (4 replicates per treatment group). Frequency refers to how many of the 4 samples plated out resulted in *Trichoderma* spp. growth.

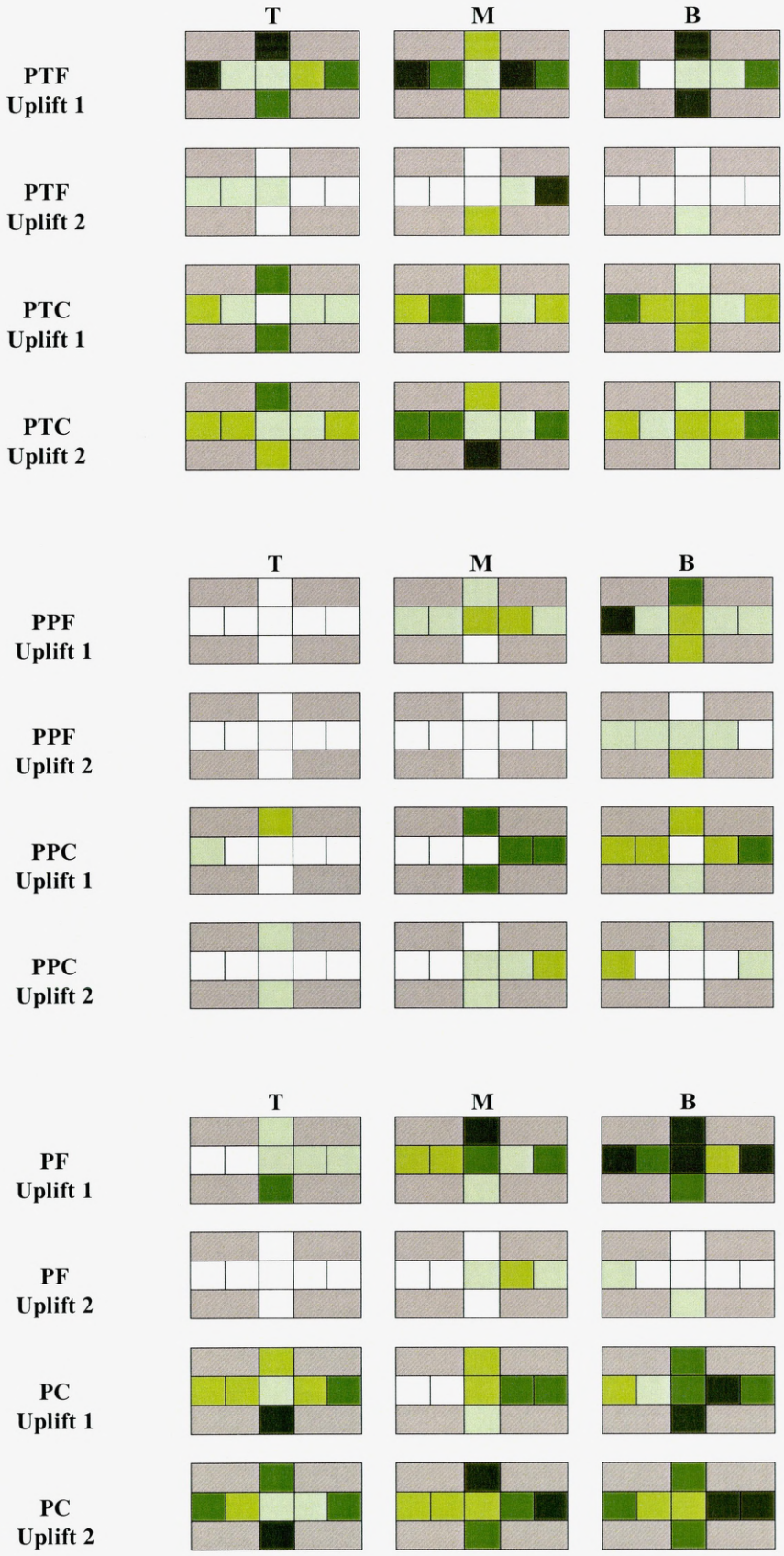


Figure 4.12 (b): Frequency of *Trichoderma* isolation from pine stakes

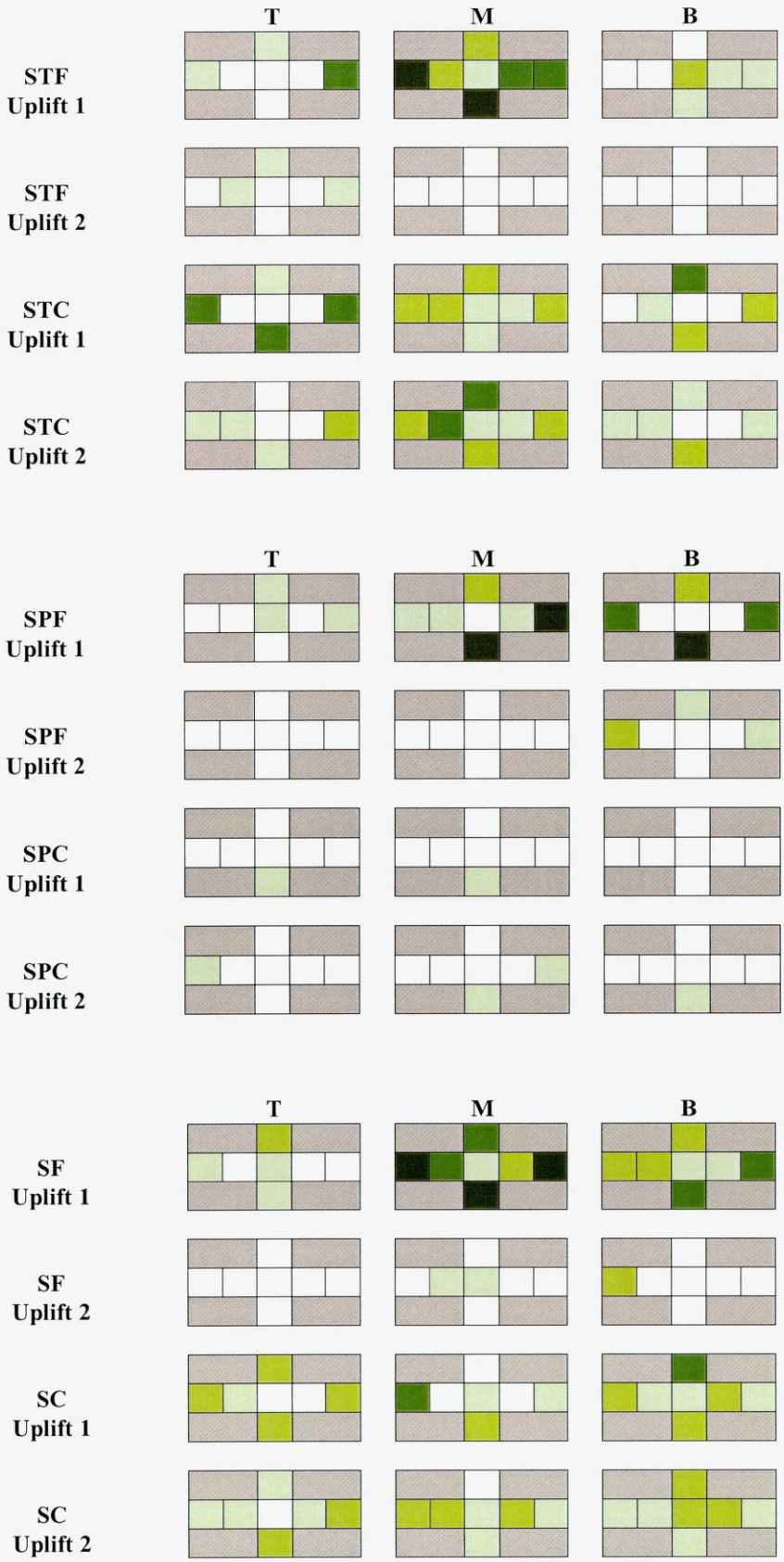


Figure 4.12 (c): Frequency of *Trichoderma* isolation from spruce stakes

4.3.3.4 Determination of moisture content

Table 4.8 (a-d) presents the moisture contents of wood blocks cut from field and fungal cellar stakes, which were calculated after the blocks had been oven-dried and weighed. These were determined both along the length (top, middle and bottom sampling points) and through the stake (cross-section blocks 1-7). Pine stakes uplifted after 9 months in the field showed clear moisture gradients top to bottom, and the moisture content results of the three treatment groups (T60, CCA and untreated controls) are statistically different from each other at all positions (top, middle and bottom) with CCA-treated pine much drier than T60-treated and untreated wood (see Appendix D (iii), Table 4.8). After 18 months in the field, all pine treatment groups had significantly higher moisture contents than the corresponding uplift 1 stakes at all positions (see Appendix D (i), Table 4.8). Moisture contents of CCA-treated wood were statistically lower, both above ground and at the groundline, with T60-treated and untreated pine controls having similar moisture contents at those positions; however there were no statistical differences between treatment groups below ground (see Appendix D (iii), Table 4.8).

Spruce stakes sampled after 9 months in the field demonstrated a similar moisture gradient to that observed in pine (Table 4.8 (c)), though T60-treated spruce had a particularly high below ground moisture content in relation to groundline and above ground moisture contents. Although CCA-treated spruce stakes were significantly wetter than either T60-treated or untreated spruce at the top and the groundline, the elevated below ground moisture content of T60-treated spruce means that both CCA-treated and T60-treated spruce are significantly wetter than the untreated spruce controls (see Appendix D (iii), Table 4.8). Significant increases in the moisture contents of spruce stakes were recorded after 18 months in the field (in comparison to uplift 1; see Appendix D (i), Table 4.8) with the exception of below

ground in untreated spruce. The untreated controls also remained significantly drier below ground than either CCA-treated or T60-treated stakes - the results in Table 4.8 (c) indicate a 4-fold increase in the below ground moisture content of T60-treated spruce from this uplift compared with the untreated spruce controls.

After 9 months in the fungal cellar, T60-treated stakes of both pine and spruce were already showing increased moisture contents and a widened range of moisture along the lengths of the stakes, in comparison to non-T60 treated wood (see Table 4.8, (b) and (d)). This is thought to be a result of T60 colonisation, with all sampling positions in T60-treated stakes being significantly wetter than either CCA-treated or untreated wood (see Appendix D (iii), Table 4.8). Moisture contents of CCA-treated spruce were markedly higher than those of corresponding CCA-treated pine stakes, particularly at the groundline and below (i.e. areas of the stakes in ground contact).

As was recorded in fungal cellar samples from uplift 1, T60-treated stakes of both wood species uplifted after 18 months were significantly wetter than stakes from either of the other two treatment groups, with T60-treated stakes displaying an exaggerated gradient range compared with CCA-treated and untreated wood (Table 4.8 (b) and (d)). This again indicates an idiosyncratic effect of pre-treatment with T60 spores on wood moisture movement and retention. T60-treated pine samples after 18 months in the fungal cellar were significantly wetter throughout the stakes than either CCA-treated or untreated pine from the same uplift (see Appendix D (iii), Table 4.8), although moisture contents at the groundline and below ground areas of T60-treated and CCA-treated pine were not significantly higher than corresponding measurements from uplift 1 (see Appendix D (i), Table 4.8). As with T60-treated pine, T60-treated spruce uplifted after 18 months in the fungal cellar was significantly wetter than the corresponding controls (both untreated and CCA-treated spruce), particularly at the groundline and below. Although the standard deviations appear to be relatively high,

the moisture content values are still significantly different (at $p \geq 0.05$). The results in Table 4.8 (d) also show that the uplift 2 moisture contents of all but 1 of the treatment groups are statistically different from each other and are all statistically significantly different from the moisture contents measured after 9 months (see Appendices D (i) & (iii), Tables 4.8). However, moisture contents recorded for fungal cellar spruce after 18 months showed an excessive increase in below ground moisture in these stakes in relation to uplift 1 moisture contents, even in untreated spruce. This effect was less pronounced in pine than in spruce, which may be due to the density differences between the two wood species.

Spruce stakes from all treatment groups tested after 18 months in the fungal cellar displayed lower moisture contents above ground than after 9 months and although this difference is not significant in T60-treated spruce, it is statistically significant in CCA-treated and untreated spruce stakes (see Appendix D (i), Table 4.8). This effect was also noted in CCA-treated and untreated pine stakes uplifted after 18 months in the fungal cellar, although T60-treated pine showed a much higher above ground moisture content than any other category. As this pattern was not evident in field stakes, it appears to have been a result of the fungal cellar environment, despite attempts to maintain the accelerated decay facility at maximum relative humidity.

Uplift 1				Uplift 2		
PTF	PPF	PF		PTF	PPF	PF
18.1 (0.8)	15.0 (0.6)	16.4 (1.0)	top	46.2 (23.7)	21.7 (0.7)	46.3 (14.5)
56.2 (24.1)	23.2 (3.6)	41.3 (10.6)	middle	91.3 (28.7)	58.9 (24.0)	91.1 (25.6)
66.5 (20.0)	40.0 (21.8)	56.1 (15.2)	bottom	89.1 (21.0)	87.7 (38.1)	80.4 (20.3)

Table 4.8 (a): Pine stakes from field tests

Uplift 1				Uplift 2		
PTC	PPC	PC		PTC	PPC	PC
45.3 (27.5)	23.1 (0.9)	22.6 (1.3)	top	71.4 (31.9)	20.3 (0.9)	18.7 (0.7)
114.3 (0.1)	28.6 (0.9)	35.9 (4.4)	middle	106. 8 (36.5)	28.9 (0.9)	53.0 (22.0)
108.8 (14.4)	39.6 (9.8)	34.3 (5.9)	bottom	108.6(28.4)	35.4 (7.3)	78.4 (34.2)

Table 4.8 (b): Pine stakes from cellar tests

Uplift 1				Uplift 2		
STF	SPF	SF		STF	SPF	SF
13.9 (0.7)	16.7 (0.6)	14.2 (0.7)	top	23.0 (0.5)	22.2 (1.3)	26.7 (4.4)
24.9 (12.3)	53.8 (19.1)	36.9 (15.4)	middle	99.4 (49.1)	95.6 (31.1)	69.0 (35.9)
115.0 (50.5)	111.8 (39.0)	40.0 (33.5)	bottom	192.5 (32.8)	155.2 (31.0)	48.2 (20.6)

Table 4.8 (c): Spruce stakes from field tests

Uplift 1				Uplift 2		
STC	SPC	SC		STC	SPC	SC
43.0 (22.2)	27.0 (0.8)	24.6 (0.8)	top	33.2 (21.1)	17.4 (1.3)	16.9 (1.4)
92.8 (52.0)	41.0 (6.3)	35.0 (4.3)	middle	149.7 (75.8)	32.9 (8.8)	46.1 (25.8)
157.3 (61.6)	80.4 (34.2)	45.6 (16.2)	bottom	262.8 (42.6)	128.1 (27.1)	183.2 (73.7)

Table 4.8 (d): Spruce stakes from cellar tests

Table 4.8: Mean moisture contents (%) for each treatment group measured by dry weight analysis. Figures in parentheses represent standard deviations and each value represents a mean of 28 readings (7 sample locations on four replicate stakes, as described in 4.2.4.3). Statistical analyses of these results are presented in Appendix D (i-iii).

4.3.3.5 Soft rot analysis

Table 4.9 (a-d) presents soft rot indices recorded for the field trial and fungal cellar stakes after 9 months and 18 months (uplifts 1 and 2, respectively). Stakes were prepared and analysed using polarised light microscopy as described in section 4.2.4.5, then by evaluating the degree of soft rot degradation in 10 fiberised wood tracheids per sample and assigning each a score out of 5 , the total score out of 50 was derived. Soft rot indices were established for each sampling depth by calculating the mean scores of 12 replicates (i.e. 3 sampling positions per stake, 4 stakes per treatment group).

The results show that after 9 months in the field, pine stakes pre-treated with the biocontrol agent displayed markedly less soft rot degradation than untreated controls at corresponding depths (Table 4.9 (a)). Although T60-treated pine stakes uplifted after 18 months also had a lower mean soft rot index than the equivalent untreated controls, variability in the extent of soft rot between samples within treatment groups has resulted in higher standard deviations. As a result, no protective effect by the biocontrol agent could be ascertained in T60-treated pine field stakes over time. Lower mean soft rot scores were recorded in T60-treated spruce stakes uplifted from the field site after both uplifts, in relation to the corresponding untreated controls; however as with the pine stakes, the high degree of variability between individual stakes masks any potential protective effect (Table 4.9 (c)). Evaluation of digested wood samples from CCA-treated field stakes revealed a very small amount of soft rot activity, present only in the outer-most 1mm samples. There were no visual indications of soft rot damage in CCA-treated stakes examined during subjective assessment, and no further soft rot degradation was observed in the next layer of tracheids (3mm).

After 9 months in the fungal cellar, soft rot scores obtained from T60-treated pine stakes were very similar to the untreated pine controls, with no evidence of the protective effect observed in field pine stakes (Table 4.9 (b)). Pine stakes uplifted from the cellar after 18 months indicated reduced soft rot in the 5mm and 7mm-depth samples from T60-treated pine compared with untreated pine, however as with the field stakes, the high stake-to-stake variability within the treatment groups makes the assessment of a definite reduction in soft rot decay impracticable. *Trichoderma*-treated spruce stakes uplifted and tested after 9 months in the fungal cellar did not demonstrate reduced soft rot attack in comparison to untreated spruce, however soft rot indices derived from T60-treated spruce stakes after 18 months in the cellar indicated an apparent reduction in the amount of soft rot activity detected in deeper samples (5mm and 7mm), despite the chronic variability and high standard deviations (Table 4.9 (d)). As with field samples, very little soft rot was evident on CCA-treated stakes, and soft rot cavities were only observed in surface samples (Table 4.9 (b) and (d)).

Although partial protection from soft rot degradation was observed in some T60-treated stakes, overall there was little evidence of predictable, lasting protection against soft rot degradation by the applied biocontrol agent. The results do however draw specific attention to the effect of wood species and exposure conditions on the behaviour and efficacy of potential biocontrol agents. In field stakes, an initial reduction in the extent of soft rot decay was observed in T60-treated pine, however this effect was no longer clear in later samples. Conversely, no protective effect was observed in T60-treated fungal cellar stakes from the first uplift, yet after 18 months T60-treated spruce demonstrated less soft rot degradation than untreated spruce. Definitive interpretations of the results of soft rot analysis were adversely affected by high variability between individual stakes in the same treatment group.

Uplift 1				Uplift 2		
PTF	PPF	PF	depth	PTF	PPF	PF
7.8 (2.6)	1.6 (3.6)	14.5 (4.2)	1 mm	14.58 (7.4)	1.0 (1.4)	22.0 (11.4)
2.1 (1.9)	0 (0)	1.5 (1.0)	3 mm	9.82 (6.6)	0 (0)	14.2 (5.23)
0 (0)	-	1.0 (1.4)	5 mm	3.55 (2.9)	-	5.4 (2.8)
-	-	-	7 mm	-	-	-

Table 4.9 (a): Pine stakes from field tests

Uplift 1				Uplift 2		
PTC	PPC	PC	depth	PTC	PPC	PC
42.9 (4.3)	1.7 (1.9)	41.9 (4.9)	1 mm	44.0 (2.9)	2.0 (3.0)	44.0 (5.6)
23.3 (10.3)	0 (0)	20.9 (9.8)	3 mm	23.6 (11.4)	0 (0)	29.3 (9.8)
10.8 (4.7)	-	11.9 (6.2)	5 mm	12.8 (7.3)	-	19.4 (8.6)
3.8 (4.6)	-	6.5 (3.9)	7 mm	6.8 (5.3)	-	10.8 (5.5)

Table 4.9 (b): Pine stakes from cellar tests

Uplift 1				Uplift 2		
STF	SPF	SF	depth	STF	SPF	SF
6.9 (4.1)	0.3 (0.8)	13.3 (5.5)	1 mm	7.1 (8.1)	0.2 (0.4)	16.2 (11.5)
1.7 (2.1)	0 (0)	3.6 (3.6)	3 mm	2.6 (2.1)	0 (0)	7.2 (4.3)
0 (0)	-	0.9 (1.5)	5 mm	0 (0)	-	1.1 (2.9)
-	-	-	7 mm	-	-	-

Table 4.9 (c): Spruce stakes from field tests

Uplift 1				Uplift 2		
STC	SPC	SC	depth	STC	SPC	SC
34.8 (7.5)	1.0 (1.3)	34.3 (10.3)	1 mm	38.2 (5.1)	0 (0)	41.9 (6.5)
23.4 (6.4)	0 (0)	17.4 (4.9)	3 mm	22.8 (14.7)	0 (0)	28.1 (6.1)
10.2 (5.4)	-	7.9 (3.2)	5 mm	7.4 (5.0)	-	16.3 (5.5)
0.3 (1.1)	-	4.3 (2.2)	7 mm	2.4 (2.0)	-	8.3 (5.8)

Table 4.9 (d): Spruce stakes from cellar tests

Table 4.9: Total mean soft rot index for field and fungal cellar stakes. Score based on assessment of 10 tracheids per sample, 3 samples per stake. (Standard deviations from three sites on four stakes (12 replicates) are presented in parenthesis).

The symbol - indicates that no tracheids were sampled from this position.

4.3.3.6 Determination of nitrogen content

Table 4.10 (a-d) presents the results of nitrogen analysis of wood samples taken from the groundline area of field and fungal cellar test stakes. After 9 months in the field, nitrogen contents of CCA-treated and untreated pine stakes (Table 4.10 (a) were very similar to the baseline nitrogen level of pine (Table 4.10 (e)). The nitrogen content of T60-treated pine measured after 9 months was lower than both the laboratory control and the two other treatment groups (CCA-treated and untreated controls), however after 18 months the nitrogen contents of all pine treatment groups had increased; T60-treated pine and untreated pine had similar nitrogen levels, while the nitrogen content of CCA-treated pine in particular was markedly higher. Spruce field stakes uplifted after 9 months displayed the same trend as pine field stakes from uplift 1 (Table 4.10 (a) and (c)), although CCA-treated and untreated spruce samples had slightly higher nitrogen contents than the spruce laboratory control, and the nitrogen content of T60-treated spruce was marginally lower than the baseline level. Nitrogen contents increased in spruce field stakes between uplifts 1 and 2, although there was less variability in the nitrogen levels between spruce stakes from different treatment groups than in corresponding pine treatment groups.

Pine stakes uplifted after 9 months in the fungal cellar displayed a much wider spread of nitrogen levels between treatment groups than field samples (see Table 4.10 (b)). CCA-treated pine stakes had nitrogen contents similar to those of unexposed laboratory control pine, however T60-treated pine had around double the nitrogen content of the baseline control and the nitrogen content recorded for untreated pine stakes was especially high in comparison to both CCA-treated and T60-treated pine. After 18 months in the cellar the nitrogen content of CCA-treated pine was noticeably higher than the corresponding uplift 1 value, and T60-treated pine had risen slightly, making the nitrogen contents of the two treatment groups almost the same. The

untreated pine control stakes displayed nitrogen contents three times higher than those of CCA-treated and T60-treated pine. Nitrogen analysis of spruce stakes removed from the cellar after 9 months showed that the nitrogen contents of all three treatment groups were distinctly higher than the baseline spruce controls (Table 4.10 (d)); T60-treated spruce displayed an elevated nitrogen content in comparison to samples from other treatments, however the high standard deviations in nitrogen values of T60-treated spruce stakes indicate that differences between individual stakes were much more pronounced in this treatment group than in CCA-treated and untreated spruce. After 18 months in the fungal cellar, the nitrogen contents of spruce stakes did not show the same pattern of elevation as pine stakes or field trial spruce stakes, and although the uplift 2 nitrogen levels of both T60-treated and CCA-treated spruce were lower than uplift 1 nitrogen levels, a high degree of variability is again evident.

In general, nitrogen contents of the field stakes displayed a clear trend, with the three treatment groups (T60, CCA and untreated) generally quite similar to one another. Increases in nitrogen levels over time were more obvious in spruce field stakes than pine however the variability observed in cellar samples, particularly spruce, made determining any common pattern difficult.

Uplift 1		
PTF	**PPF**	**PF**
0.09 (0.02)	0.13 (0.01)	0.13 (0.02)

Uplift 2		
PTF	**PPF**	**PF**
0.16 (0.06)	0.27 (0.21)	0.17 (0.02)

Table 4.10 (a): Pine stakes from field tests

Uplift 1		
PTC	**PPC**	**PC**
0.27 (0.06)	0.18 (0.03)	0.65 (0.06)

Uplift 2		
PTC	**PPC**	**PC**
0.32 (0.04)	0.31 (0.03)	1.04 (0.08)

Table 4.10 (b): Pine stakes from cellar tests

Uplift 1		
STF	**SPF**	**SF**
0.11 (0.01)	0.14 (0.01)	0.14 (0.01)

Uplift 2		
STF	**SPF**	**SF**
0.20 (0.04)	0.22 (0.07)	0.21 (0.01)

Table 4.10 (c): Spruce stakes from field tests

Uplift 1		
STC	**SPC**	**SC**
0.61 (0.11)	0.25 (0.06)	0.40 (0.05)

Uplift 2		
STC	**SPC**	**SC**
0.50 (0.15)	0.20 (0.07)	0.63 (0.03)

Table 4.10 (d): Spruce stakes from cellar tests

pine	spruce
0.14 (0.02)	0.12 (0.01)

Table 4.10 (e): Control samples of pine and spruce for comparison

Table 4.10: Mean nitrogen content (%) measured at the groundline region of field and fungal cellar stakes. Means are of 8 samples per group (2 per stake). Figures in parentheses are standard deviations.

4.4 Discussion

The results presented in section 4.3 are the outcome of detailed biological testing of wood pre-treated with a potential biocontrol agent, *Trichoderma viride* isolate T60, as an assessment of the biological control capacity of the organism under field conditions. The field trial was designed using European Standard EN 252 (pertaining to field testing of chemical wood preservatives) as a framework. In addition to the subjective assessment of test specimens following exposure to soil contact recommended in the standard, a number of biological examinations were also carried out. These analyses were selected to provide information about the activity of the biological control agent against other wood-colonising micro-organisms, particularly decay fungi, in situations more closely resembling the end use of ground contact timber. Biological assessments of field and fungal cellar test stakes indicated marked effects on the behaviour and activity of the selected *Trichoderma* isolate observed in different environmental conditions (i.e. field site or fungal cellar) and wood species.

The design and set up phases of the field and fungal cellar tests in this study were described in Brown and Bruce (1999), which also reported the initial results of sapstain and moisture monitoring. Analyses of laboratory assessment of the first uplift of stakes, after 9 months in the field and fungal cellar, were reported in Brown *et al.* (1999) and the results indicated a definite effect on the patterns and extent of decay recorded in T60-treated wood. The effects of pre-treatment with *Trichoderma* spores on the moisture contents of pine and spruce were also evident, even after a relatively short exposure period (Brown and Bruce, 1998; Brown *et al.*, 1999).

Moisture Content

Monitoring of soil and wood moisture levels over the duration of the field trial test period provided information regarding the environmental conditions to which the stakes were exposed, and gave preliminary impressions of the effect of biological control agents on moisture contents in ground contact timbers. Wood moisture contents recorded over time (Figure 4.8 (a-f)) demonstrated that the differences between moisture contents above ground and at the groundline were less defined in fungal cellar stakes than in field stakes, due to a combination of higher soil moisture content (as shown in Figure 4.7) and higher relative humidity in the accelerated decay testing environment. The charts presented in Figure 4.8 also indicated that stakes from the fungal cellar generally had higher moisture contents overall than field stakes. Moisture contents of cellar stakes were found to be consistently above fibre saturation point, whereas the moisture contents of field stakes were more variable as a result of seasonal (and daily) fluctuations in temperature and moisture; Figure 4.8 illustrates the different moisture patterns observed over time between the two test systems (field and fungal cellar). The influence of soil moisture content on the moisture content of wood in ground contact was also observed in virulence control blocks used to assess the decay capacity of the soil in the fungal cellar. These results, i.e. soil moisture content (shown in Figure 4.7) and moisture contents of fungal cellar virulence controls (Figure 4.9 (b) and Table 4.2), indicated that tub-to-tub variations in soil moisture content were reflected in the wood moisture content of fungal cellar samples.

A moisture meter was used to measure surface moisture content of test stakes immediately following uplift to assess whether wood at the stake surfaces was above fibre saturation point (FSP), as availability of free water within the wood cell is an important factor governing fungal colonisation of wood as a substrate. Wood with a

moisture content below 20% is essentially immune to decay fungi (Sinclair, 1995), however when the moisture content rises above 25 - 30% (i.e. FSP), wood is considered to be at risk from attack by decay fungi (Eaton and Hale, 1993). Surface moisture measurements presented in Table 4.4 show a recurring pattern of moisture distribution along the length of test stakes, and above ground moisture contents were generally much lower than moisture contents measured at positions previously in ground contact (i.e. groundline and below ground.).

The results in Table 4.4 also show that while field trial stakes of both wood species had similar moisture contents, spruce cellar stakes were much wetter than corresponding pine cellar stakes. In general, higher moisture contents were recorded in cellar stakes than in field stakes which is consistent with the results of wood moisture monitoring presented in Figure 4.8. It is generally accepted that there is a certain limitation of accuracy associated with the use of capacitance-measuring moisture meters when wood is above certain moisture contents. This is particularly pertinent in wood treated with ionic salt formulations such as CCA, due to the effects of preservative salts on wood conductivity. However, the results presented in Table 4.4 indicate that as moisture contents recorded at areas of the test stakes in ground contact were generally at or above fibre saturation point, moisture conditions in both the field and fungal cellar test systems were favourable for fungal colonisation and wood decay (Morrell and Gartner, 1998).

The results of preliminary moisture assessment (Table 4.4) correlate well with the results of pilodyn testing to measure the resistance to impact of test stakes, which are presented in Table 4.5. After 9 months exposure, differences between the surface softness measured above the groundline and the surface softness of areas previously in ground contact were mostly attributed to a combination of relatively minor surface

soft rot degradation (in ground contact areas), and differences in the surface moisture content of the wood measured by electrical conductivity (Table 4.4); the low levels of decay detected in uplift 1 samples could not solely account for increased surface softness. Although Friis-Hansen (1980) reported that moisture contents above FSP had a limited effect on pilodyn testing and Hainey (1992) observed that wood moisture content had a negligible impact on pilodyn readings, it appears that the lower pin-propelling force and smaller stake dimensions used in this study resulted in moisture content and wood species having a more pronounced effect on the measurement of surface softness using a pilodyn.

The results of whole stake analysis moisture content determination (Table 4.7) revealed extremely high moisture contents in T60-treated wood in relation to moisture contents recorded in other treatment groups. Moisture contents measured in field stakes after 9 months were the lowest overall, whereas the highest moisture contents were measured in cellar stakes. As only one stake from each treatment group could be assessed in this way, statistical significance of these results in relation to one another was not determined. The results in Table 4.7 demonstrated less direct correlation between moisture content and weight loss than anticipated, however differences between moisture contents of stakes from corresponding treatment groups appeared to follow specific trends defined by wood species and test conditions.

The moisture contents presented in Table 4.8 were determined by oven-dry weight analysis and demonstrate a high degree of variability within treatment groups, due in part to inter- and intra-stake differences in moisture. A degree of variability was expected due to the method of block sampling used; i.e. measuring the moisture content of the 7 individual blocks from within 3 cross-sections taken from each of 4 stakes, then pooling the values obtained to determine a mean moisture content value per position (top, middle or bottom) per treatment group (T60, CCA, control). This

was done in order to determine the extent of changes in moisture content throughout the stakes and the high standard deviations calculated for some values are indicative of the changes in moisture content through the cross-sections. Differences between soil moisture contents of individual soil beds in the fungal cellar further increased the variability of moisture levels observed in cellar samples, thus also increasing standard deviation values.

Moisture contents of field trial stakes uplifted after 9 months indicated that spruce field stakes displayed different moisture patterns than corresponding pine stakes. Although CCA-treated pine stakes from the field were drier than T60-treated and untreated wood, and T60-treated pine was the wettest of the 3 pine treatment groups (Table 4.8 (a); Appendix D (iii), Table 4.8), CCA-treated spruce was wetter at the groundline than the other treatment groups. T60-treated spruce appeared to be abnormally dry at the groundline, particularly given the recorded below ground moisture content in the same stakes (Table 4.8 (c)). After 18 months exposure untreated field stakes had much higher moisture contents at the groundline than in uplift 1, indicating that degradation was occurring in areas of untreated wood in soil contact. The wood/soil interface is particularly susceptible to microbial degradation due to the occurrence of favourable decay conditions (Levy and Dickinson, 1981). Simultaneous availability of several factors essential to growth, i.e. the accessibility of moisture, nutrients and oxygen at the groundline of timber in ground contact, encourages the activity of wood-colonising soil fungi.

Moisture contents recorded for cellar stakes demonstrated that even after 9 months, T60-treated wood was excessively wet at and below the groundline in comparison to other treatment groups of both wood species, however CCA-treated and untreated pine stakes displayed lower, less variable moisture contents than the corresponding spruce stakes (Table 4.8 (b) and (d); Appendix D (iii) Table 4.8).

CCA-treated pine uplifted from both the field and the cellar was consistently drier than T60-treated wood (Table 4.8 (a) and (b)); CCA-treated wood has been reported previously to have lower moisture contents than untreated wood (Gray, 1986; Green, 1988) as the active compounds in the preservative bind with hydroxyl groups in the wood structure during fixation, reducing the amount of bound water the wood can absorb.

As observed in uplift 1, the moisture contents of spruce stakes removed from the cellar after 18 months were highly variable (indicated by the elevated standard deviations), particularly in areas of ground contact (Table 4.8 (d)). Although T60-treated stakes were very wet, the variability encountered does not appear to be due to treatment (i.e. CCA, T60 or untreated) as much as wood species (Appendix D (iii) Table 4.8). Hainey (1992) reported that the wide variability of moisture contents measured in spruce and pine pole sections exposed to soil contact was dependent on two factors: wood species, and the presence or absence of CCA treatment. Spruce is a refractory timber and is difficult to impregnate with chemical preservatives, due to both closure of the pit membranes during drying (aspiration), and anatomical characteristics. Spruce species have smaller pits than pine species, and a smaller proportion of ray tracheids which are also narrower than those in pine wood (Hainey, 1992). Ray tracheids and pit apertures are key avenues of fluid conduction in most wood species (Morrell and Morris, 2002) and it is therefore possible that as a result of pit aspiration and wood structure, moisture movement is more erratic in spruce than in non-refractory wood species.

Excessive moisture contents have been shown to inhibit microbial degradation of wood, for instance the use of ponding to reduce biodeterioration of logs. However, untreated spruce control stakes in this study demonstrated high moisture contents with no indications of decay inhibition or retardation. This indicates that any

reduction in biodegradation observed in T60-treated wood was due to the action of the applied biocontrol agent, rather than being a result of the wide-ranging variability recorded in moisture contents of *Trichoderma*-treated wood. However, high levels of wood decay (as seen in the untreated spruce cellar samples) will affect the outcome of moisture contents determined by dry weight analysis. Hainey (1992) observed that high moisture levels recorded at the surface of untreated spruce and pine were associated with the presence of soft rot decay.

Pre-treatment of wood with *Trichoderma* spores also appeared to have a marked effect on the moisture contents of stakes, with moisture levels as much as 4 times higher than corresponding untreated controls recorded in some instances. T60-treated pine from the cellar was found to be much wetter than untreated pine after both time periods. These excessive increases generally occurred in areas of the test stakes previously in direct soil contact, and the pattern of the moisture increases in T60-treated wood in comparison to CCA-treated or untreated wood suggests that colonisation by the biocontrol agent may have increased the potential for lateral moisture uptake and movement in addition to vertical moisture translocation. This may be a result of the cellulolytic and pectinolytic action of *Trichoderma* spp. enzymes, degrading the tori of pit membranes and thereby increasing the permeability and moisture-uptake capabilities of the wood (Johnson and Gjovik, 1970). Enzymatic opening of the pit membranes has been investigated as a way of countering the problems caused to wood treaters by refractory species, and the application of certain wood-colonising fungi to logs prior to pressure-impregnation has been shown to increase the porosity of wood, resulting in improved penetration of preservatives in subsequent treatment. (Rosner *et al.*, 1998; Tucker *et al.*, 1998). However, wood colonised by moulds may also bleed applied preservatives while in service (Morrell

and Morris, 2002), indicating that the effects of permeability enhancement on subsequent moisture conduction in wood are not, as yet, predictable or fully defined.

Colonisation

The results presented in this chapter illustrate the challenge of applying biological control agents for the protection of ground contact timber from decay fungi. A number of factors can affect the behaviour and antagonistic potential of the selected biocontrol agent, such as temperature, humidity and nutrient availability, for target fungi as well as antagonists (Hjeljord and Tronsmo, 1996). The results of the field and fungal cellar trials reflected the influence of the different environmental conditions that the stakes were exposed to on the activity of the biocontrol agent, and highlighted the effect of wood species on biocontrol potential.

Wood used in this field trial was treated with T60 spores (suspended in sterile water) or the reference wood preservative CCA using pressure impregnation regimes recommended for the chemical preservation of sawn wood (Chapter 3). Subsequent germination of *Trichoderma* on the surface of T60-treated stakes confirmed that biocontrol agents could be applied using standard industry processes, and that nutrient-supplemented treatment solutions are not essential to colonisation of the substrate. Score (1998) reported that *Trichoderma harzianum* isolate T25, applied as a spore suspension in sterile water and sprayed onto *Serpula lacrymans*-infected wood, did not colonise the treated areas due to inadequate nutrient availability. As previously noted, moisture availability is also an important consideration when designing a delivery system. Philp (1998) stated that *Trichoderma* spores applied to wood as surface coatings or pellets did not germinate and fully colonise Sitka spruce and Scots pine logs because the moisture content of the substrate was too low.

Wood slivers were plated out onto TSM selective media as described in section 4.2.4.3 in order to isolate *Trichoderma* spp. from field trial and fungal cellar stakes. In both field and cellar test stakes, the frequency of *Trichoderma* isolation from spruce stakes was lower than isolation from pine samples, in both pre-treated and untreated wood (Figure 4.12). Scots pine has a higher proportion of soluble nutrients than Sitka spruce (Nayagam, 1987) and the higher frequency of *Trichoderma* isolation from pine stakes is a direct reflection of the nutritional status of pine in comparison to that of spruce (Breuil, 1998). This is further verified by other results reported in this chapter, in particular the rapid discoloration of pine by sapstain organisms in comparison to spruce (Brown and Bruce, 1999).

The isolation of *Trichoderma* spp. from field and fungal cellar stakes was expected to show a higher frequency of isolation from stakes pre-treated with T60 spores. However, the results of the non-specific isolation using TSM demonstrated equivalent frequencies of *Trichoderma* isolation from untreated wood compared with T60-treated wood. These results illustrate the variability between the antagonistic properties of different strains of *Trichoderma* spp. in that despite abundant isolation from untreated wood, no protection was apparent in untreated controls, whereas stakes pre-treated with T60 spores exhibited differences in the levels of degradation observed compared with the untreated controls.

The results presented in Figure 4.12 also highlight a particular concern with regard to the use of biological control agents to protect ground contact timbers, the need for continuing viability of applied organisms. Isolation of *Trichoderma* spp. from field stakes of both wood species was considerably reduced after 18 months, in relation to the isolation frequencies of corresponding stakes from uplift 1 (9 months). The associated decrease in the amount of *Trichoderma* present in field stakes over time, particularly in pre-treated wood, raises questions about the long term viability

of applied biocontrol agents. Fungal cellar stakes did not exhibit the same trend as field stakes, in that the frequencies of isolation after 18 months were equivalent to those after 9 months in corresponding stakes. As the length of exposure time was the same for both field trial and fungal cellar stakes (a maximum of 18 months), it is unlikely that the observed reduction in isolation was due to nutrient limitation, i.e. the consumption of all available nutrients in the wood. Therefore, the most obvious explanation for these results is the influence of environmental conditions on *Trichoderma* growth; stakes undergoing testing in the fungal cellar were exposed to constant, warm conditions in a high relative humidity, and so the frequency of *Trichoderma* isolation from cellar stakes was more consistent between uplifts. Although the results could be an indication that the applied biocontrol agent would not survive for a prolonged period under the environmental conditions in the field, it is not the only possible explanation for the reduced isolation frequencies. As the same pattern was observed in field stakes regardless of wood species or treatment group (i.e. T60-treated, CCA-treated or untreated controls), changes in temperature and available moisture over time could have resulted in a seasonal latency of the overall microbial population at the field site, particularly as field uplift 1 took place in April (spring) and uplift 2 in January (during winter).

Trichoderma spp. were also isolated from CCA-treated wood, but at a much lower frequency than in non-preservative treated wood. Being a water-borne formulation, CCA has a different mode of action from that of oil-based wood preservatives such as creosote and does not act as a repellent in the same way (Mowe, 1983). Therefore, while some fungi may be present in CCA-treated wood, they are unlikely to colonise the wood extensively or cause significant decay.

Overall, despite the similarities in *Trichoderma* isolation frequencies between T60-treated and untreated wood, a protective effect was observed in pre-treated stakes

that was not evident in untreated controls, indicating that specific isolates of *Trichoderma* spp. vary greatly in antagonistic properties and mechanisms of action (Hjeljord and Tronsmo, 1996). The effect of T60 colonisation of pre-treated wood was also evident in the moisture content results discussed previously; T60-treated stakes demonstrated excessive increases and variability in moisture contents, but this effect was not observed in untreated controls regardless of the amount of non-applied *Trichoderma* spp. isolated. This again indicates variability in activity between different isolates of *Trichoderma*, particularly in their capacity to produce degradative enzymes.

Pre-treatment with *Trichoderma* spores was also shown to have had a marked effect on the rate of sapstain colonisation of pine stakes in the field, which was lower than the rate at which discoloration appeared on the corresponding untreated pine controls (Brown and Bruce, 1998; 1999). This effect was not apparent in spruce stakes in which sapstain development, though slower than in pine, was similar in both T60-treated stakes and untreated spruce controls. Overall, sapstain colonisation in cellar stakes was slower and generally less severe than in field stakes. This is most likely to have been a due to the closed, controlled environment of the fungal cellar, resulting in a marked reduction in the wind-borne and insect-borne spread of sapstain.

Certain species of wood such as pine are particularly prone to attack by staining fungi, while spruce species are less susceptible (Breuil, 1998) and this may be due to differences in nutrient provision. As mentioned previously in relation to *Trichoderma* colonisation, pine has a higher nutrient status than spruce (Nayagam, 1987) and this may explain the increased rate of sapstain colonisation observed in the untreated pine stakes. Staining fungi produce lipases to hydrolyse wood triglycerides into fatty acids and glycerol for use as nutrients for fungal growth (Breuil, 1998), in addition to utilising sugars and nitrogen. The observed biocontrol in the

Trichoderma-treated pine stakes may be a result of competition for those nutrients between the inoculated organism and invading fungi, thereby slowing the colonisation by sapstain fungi. Competition for nutrients is one of a number of ways in which *Trichoderma* spp. are thought to inhibit the growth of other wood-colonising fungi. The apparent lack of protection in the spruce stakes may be a result of lower nutrient status of spruce in comparison to pine, meaning reduced nutrient availability for the biocontrol agent to establish its presence and compete with the staining fungi.

Sapstain is generally caused by the growth of darkly-pigmented fungal hyphae through wood cell lumens, producing a blue-black discoloration of the wood. Sapstain organisms will preferentially colonise freshly felled wood, and as such the critical stages in controlling sapstain development are immediately post-felling and during the early service life of the timber before chemical treatment or pulping of the wood takes place (Schoeman *et al.*, 1999). Therefore the ability of a biological control agent to delay the onset of fungal discoloration of wood rather than completely prevent the occurrence of such staining is particularly relevant to sapstain biocontrol, although as *Trichoderma* spp. can cause discoloration of wood during sporulation (as demonstrated in Figure 3.5), there has also been investigation into the use of non-pigmented mutant strains of potential biocontrol isolates of *Trichoderma* (Horvath *et al.*, 1995; Bruce, 1998)

The results of nitrogen analysis carried out on pooled groundline samples of field and fungal cellar stakes showed that the nitrogen content of *Trichoderma*-treated field samples are initially lower than either the CCA-treated or untreated field samples, both of which were determined to have similar nitrogen contents (Table 4.10). As increases in wood nitrogen levels can be due to either translocation of the nutrient by a colonising microbe, or increases in nitrogen-rich microbial biomass

(King *et al.*, 1980; Mowe, 1983), the relatively low nitrogen value may have been a result of the biological control agent inhibiting colonisation of pre-treated stakes by "invading" organisms such as moulds and primary decay fungi.

Overall, the results in Table 4.10 show significantly higher nitrogen levels detected in T60-treated and untreated cellar stakes than in corresponding field stakes, particularly after 18 months. This suggests increased microbial presence and activity in these stakes, particularly with regard to untreated pine removed from the fungal cellar after 18 months. Untreated pine had a significantly higher nitrogen content than untreated spruce samples from the cellar, which can be attributed to the higher nutrient status of pine (Nayagam, 1987), and to the higher amounts of soft rot and basidiomycete decay found in untreated pine from the cellar than in T60-treated or CCA-treated pine. Although the *Trichoderma*-treated pine had nitrogen contents of around half that of *Trichoderma*-treated spruce, there were no significant differences in the patterns of soft rot decay that would adequately account for this. It is possible however that higher moisture contents in the *Trichoderma*-treated spruce encouraged colonisation by other organisms therefore leading to increased microbial colonisation of the wood. Overall, higher nitrogen contents were recorded in cellar samples than in field samples, indicating a higher degree of colonisation in wood exposed to non-sterile soil than in wood from the field site, due to more favourable decay conditions. The results of preliminary decay assessments carried out on the same stakes showed little correlation with nitrogen analysis; although untreated pine controls had higher nitrogen content than spruce, the extent of degradation was worse in untreated spruce stakes than in untreated pine.

The wood preservative CCA does not act as a volatile repellent of micro-organisms in the same way as oil-borne preservatives, such as creosote. Therefore

fungi may colonise CCA-treated wood although the timber should still be protected against decay (King *et al.*, 1979). In general, the nitrogen contents of CCA-treated wood in this field trial increased over time which indicated a degree of microbial colonisation, however as there were little or no signs of biodeterioration in CCA-treated stakes, it was concluded that the wood was still adequately protected.

Overall, the nitrogen contents recorded could not be correlated directly with any of the decay or isolation experiments carried out on stakes. However, detailed analysis of nitrogen contents from sampling sites including above and below ground samples and measuring in incremental depths rather than pooled samples, might have provided more definitive information regarding localised increases in nitrogen during decay.

Observations and measurements of fungal colonisation of test stakes recorded during the field trial highlighted the effects of both the environmental conditions under which wood is assessed, and wood species on the overall performance of the selected *Trichoderma* isolate. In general, stakes tested in the fungal cellar exhibited increased fungal colonisation; the presence of *Trichoderma* spp. over time was higher and more prolonged in cellar stakes than in field trial stakes; there was also more extensive decay recorded in cellar samples than in corresponding field stakes. Wood species affected the recorded results in a variety of ways; for example, differences in wood density influenced moisture content and movement, in addition to the effect of density on resistance to impact testing; the nutrient status of the wood resulted in preferential colonisation of one wood species over the other, i.e. pine has a higher available nutrient content than spruce, and the results clearly show that pine was colonised more rapidly or to a greater extent by *Trichoderma* spp., sapstain fungi and soft rot fungi. The low frequency of basidiomycete isolation means that although the

same pattern may have been present for the colonisation of different wood species by basidiomycete fungi, no trend was identified.

Prevention of Decay

The results of subjective assessments carried out on test stakes immediately following removal from the soil are presented in Table 4.3 (a-d), and indicate that there was a moderate degree of protection evident in T60-treated wood after 18 months exposure in the field in comparison to untreated field control stakes. The mean subjective scores recorded in fungal cellar samples show that although T60-treated stakes did not appear to perform significantly better than untreated controls, impact failure of test specimens after 18 months was only observed in untreated controls and not in *Trichoderma*-treated stakes. Impact failure of a field trial stake occurs when the mechanical properties of the wood have been so badly affected by microbial colonisation and attack that the stake has no significant strength or elasticity and simply breaks. Therefore, while biocontrol-treated stakes were clearly not as well protected against decay as CCA-treated stakes, wood pre-treated with T60 spores was found to perform notably better than untreated wood in that none of the *Trichoderma*-treated stakes uplifted during the study were decayed to the point of failure. In general, the results of subjective assessment of stakes demonstrated the increased decay capacity and moisture content of fungal cellar soil in comparison to field soil conditions.

Whole stake analysis of weight losses in individual field and fungal cellar stakes showed that overall, cellar stakes experienced higher weight losses than field stakes, as expected; however, the T60-treated spruce stakes uplifted from the cellar demonstrated a marked reduction in weight loss compared to the untreated controls.

Measurement of the resistance to impact of test stakes was carried out using a pilodyn to determine differences in surface softness (Table 4.5 (a-d)). T60-treated field stakes of both wood species showed no significant difference in their resistance to impact in relation to untreated controls. Although there is no evidence of T60 preventing decay in field trial stakes, the resistance to impact of fungal cellar stakes measured after 18 months indicated that the biocontrol agent was exerting a protective effect in T60-treated cellar stakes. In spruce stakes, T60-treated wood was less softened at the groundline than untreated spruce after 18 months in the cellar. This correlates well with the subjective assessment results, where impact failure was recorded in untreated cellar stakes at uplift 2 but not in T60-treated stakes.

The effects of accelerated decay conditions on pilodyn readings were evident in that cellar stakes generally demonstrated increased surface softening compared with field stakes. However, fungal attack is not the only factor influencing resistance to impact testing; increased temperature and humidity will affect the moisture content of the wood being tested, subsequently wood surfaces will become softer and less resistant to impact. Moisture content can have a considerable effect on surface softening of wood, and although T60-treated pine had a significantly lower resistance to impact above ground than untreated pine after 18 months in the fungal cellar, surface moisture measurement results for corresponding stakes presented in Table 4.4 indicate that the differences in surface softness observed were due to elevated above-ground moisture contents as opposed to any weakening of the wood structure caused by the applied biocontrol agent. An important consideration in the application of wood colonising fungi as a biological control agent is that there cannot be any detrimental effects on wood strength properties.

The resistance to impact of the CCA-treated wood was higher than in other treatment groups and was shown in the case of CCA-treated cellar pine to be

significantly higher (Appendix D (iii) Table 4.5). The use of CCA has been shown to increase the hardness of wood (Jonnsson *et al.*, 1989) and this effect is thought to be due to the formation of rigid polymeric structures during chromium (VI) fixation with lignin and cellulose (Hainey, 1992; Pizzi, 1979; 1981) however this effect was not generally seen in the CCA-treated spruce stakes examined in this study. Although CCA treatment can result in lower wood moisture contents which could compound the effect of the preservative on resistance to impact, comparison of pilodyn results and moisture contents in CCA-treated spruce and pine (Tables 4.5 and 4.4, respectively) indicate that wood species must also be exerting an influence on the resistance to impact of CCA-treated test stakes. The results of pilodyn testing can be compared with other results showing initial reductions in decay in T60-treated pine field stakes, as reported in Brown *et al.* (1999), and reduced decay in T60-treated spruce in cellar after longer exposure. The results also indicate that it is unlikely that increased moisture contents at certain sampling points were due solely to extensive decay as this would have been accompanied by higher values when surface softness was tested using the pilodyn.

The results in Table 4.5 of resistance to impact testing showed that pine was generally harder than spruce, and pine stakes from all three treatment groups (T60, CCA and untreated) did not exhibit any significant increases in surface softening over time (Appendix D (i). As with the results of moisture content measurement, pilodyn results demonstrated that the physical effects of CCA treatment on wood were dictated, at least in part, by wood species - CCA-treated pine was harder than non-preserved wood, although the effects of chemical preservative treatment on wood hardness were not as apparent in CCA-treated spruce.

Although pilodyn testing provided a preliminary indication of the occurrence of surface softening, polarised light examination of chemically digested wood fibres

proved to be a more sensitive technique for the detection of soft rot decay (Table 4.9 (a-d)). This method also has the advantage of being less affected by external variables such as wood moisture content, wood density and preservative treatment. T60-treated stakes uplifted after 9 months in the field showed around half the amount of soft rot decay than that observed in untreated samples of pine and spruce. This correlates well with a previous laboratory-based study carried out using non-sterile soil, designed to assess the effectiveness of the potential biocontrol agents against a wide range of wood degrading micro-organisms (Brown *et al*, 1996). This earlier study found that although measurable decay was recorded in all of the test blocks, there was a significant reduction in the rate of decay in the *Trichoderma*-treated blocks (section 2.3.1).

The biocontrol agent applied to spruce and pine stakes for testing in the field and fungal cellar displayed an initial protective effect in field samples, with a distinctly lower soft rot score as soft rot degradation had not penetrated as far into T60-treated stakes. The protection against soft rot decay observed in T60-treated pine removed from the field in uplift 1 was no longer apparent in pine uplifted after 18 months in the field. However, T60-treated spruce from field uplift 2 still demonstrated reduced soft rot degradation compared with untreated controls.

Although most polarised light results from the first fungal cellar uplift did not indicate any reduction in soft rot decay of *Trichoderma*-treated stakes, markedly less soft rot was detected in T60-treated spruce than in untreated cellar controls after 18 months. Soft rot scores for T60-treated pine uplifted from the fungal cellar after 18 months were slightly lower than for corresponding untreated pine stakes, however the high degree of variability within treatment groups made it difficult to establish whether T60-treated pine was being protected.

The protective effect observed in T60-treated spruce was more pronounced after 18 months exposure in the fungal cellar than after 9 months, emphasising the effect of environmental conditions on microbial behaviour. The antagonistic properties of *Trichoderma* spp. can be affected by temperature, and conidial germination can also be adversely affected by even slight changes in relative humidity (Hjeljord and Tronsmo, 1996). Conditions in the fungal cellar have already been shown to have improved the growth and survival of *Trichoderma* spp. in test stakes (Figure 4.12), perhaps due to either the absence of cold or intemperate conditions, or the increased moisture and relative humidity in the decay facility.

Lower levels of soft rot were generally observed in spruce stakes than in pine and as with sapstain assessments and *Trichoderma* isolation, the results of soft rot analyses indicate that wood species wields a significant influence on the growth of wood-colonising micro-organisms. More colonisation and decay activity was observed in pine stakes than in spruce, and pine species have a higher available nutrient content than spruce woods (Nayagam, 1987; Breuil, 1998).

Comparatively little soft rot was observed in CCA-treated wood, as expected. Unlike oil or solvent based preservatives which release volatile compounds to repel soil microbes and prevent colonisation, wood treated with copper-chrome based formulations such as CCA may still be colonised by micro-organisms but decay will be inhibited (Mowe, 1983). Butcher and Nilsson (1982) suggested that CCA masked initiation sites for soft rot cavity formation, in addition to its biocidal activity. Although there have been reported incidences of CCA-treated stakes failing during their expected service period (Hainey, 1992; Daniel and Nilsson, 1998), such occurrences are relatively rare, and are generally attributed to either uneven

preservative penetration and protection (Hainey, 1992) or colonisation by copper-tolerant decay fungi (Gray and Dickinson, 1987; Daniel and Nilsson, 1998).

The results of soft rot decay detection, as with a number of other results obtained from field trail and fungal cellar stakes, illustrate the influence of testing environment and wood species on biocontrol efficacy and performance. Ruddick (1989) stated that the ecology of decay under accelerated conditions such as a fungal cellar may not truly represent decay under field conditions. Potentially, different populations of organisms may have been responsible for soft rot degradation in the fungal cellar and at the field site. Some of the soft rot degradation of the cellar samples was found to be more severe than in field stakes, however optimal decay conditions may simply have resulted in more aggressive decay activity by wood-degrading fungi. Although the results of fungal cellar testing in this study are not strictly representative of prolonged exposure in the field, assessment of the proposed biocontrol agent under accelerated decay conditions served to highlight the effect of environmental factors on the behaviour and long-term survival of *Trichoderma viride* isolate T60.

Another significant cause of surface softening and degradation in unprotected ground contact timbers, besides moisture content and soft rot, is colonisation and decay by basidiomycete fungi. Wood samples from field and cellar stakes were plated onto a basidiomycete-selective media, containing the mould-inhibiting compound benomyl and an antibiotic to control bacterial contamination. Although only a small number of basidiomycetes were successfully isolated, the results in Table 4.6 do show a lower frequency of basidiomycete isolation from T60-treated wood in comparison to the frequency of isolation from untreated controls.

Soil micro-organisms such as *Mucor* spp. and *Penicillium* spp. were present as contaminants in most samples, as were some bacteria, despite the use of selective compounds and surface sterilisation of wood samples prior to plating. In most instances contamination was prevalent, particularly in samples excised from areas of the stakes previously in direct soil contact. Less contamination was encountered in above ground samples, particularly in field stakes, but basidiomycetes were also less evident in these areas. Overall, the results show a low frequency of isolation from field and fungal cellar stakes due in part to heavy contamination by moulds. The fungal cultures isolated from test stakes as basidiomycetes were plated onto gallic acid media to determine the type of basidiomycete present in field trial and fungal cellar stakes, on the basis of lignolytic enzyme production (which is particular to white rot fungi) and each of the isolates tested presented as brown rot fungi.

Although there was one instance of basidiomycete isolation from a CCA-treated pine cellar stake, this was isolated as a result of sampling at pre-determined points (i.e. all stakes were sampled at eight pre-determined points, and additional samples were taken from the outer edge of decay pockets if present). The fungal isolate recovered from CCA-treated pine was not visibly associated with decay of the wood and no basidiomycete decay was observed in any of the CCA-treated stakes over the test period.

The wood preservative CCA is a water-borne solution of heavy metal ions, the active ingredients of which are biocides and thus toxic to biodeteriogens; copper acts as a fungicide and arsenic as an insecticide, while chromium is the fixative agent Mowe, 1983). Although copper is the primary fungicidal agent in copper-chrome based preservatives, the presence of arsenic in the wood may also increase protection against copper-tolerant fungal species (Hainey, 1992).

Basidiomycete fungi which demonstrate copper tolerance have been documented by a number of authors (Eaton and Hale, 1993; Gray and Dickinson, 1987; Humar *et al.*, 2002; Pohleven *et al.*, 2002), and recent research into the mechanisms of copper-tolerance in brown rot fungi has identified the role of oxalic acid in the neutralization of copper by brown rot fungi (Humar *et al.*, 2002). Oxalic acid is characteristically produced by basidiomycetes and is thought to play a crucial role in the pre-enzymatic degradation of wood (Jellison *et al.*, 1997), possibly by chelating calcium (Green III *et al.*, 1997). One mechanism by which oxalic acid is purported to detoxify copper in wood is through the formation of non-toxic copper oxalate crystals (Pohleven *et al.*, 2002). These crystals are water-insoluble, therefore the inhibitory effect of copper present in wood in a complex such as copper oxalate is greatly reduced (Humar *et al.*, 2002). It has also been demonstrated that copper-sensitive brown rot fungi are involved in the diffusion and relocation of soluble copper in wood cells, by increasing the amount of free water present (Humar *et al.*, 2002).

The isolate selected for use as a biocontrol agent in this field trial was selected on the basis of its performance in laboratory screening tests. *T. viride* isolate T60 was reported by Tucker *et al.*, (1997) to be completely effective in preventing decay of wood blocks by selected basidiomycete fungi. The results of non-sterile soil screening of T60 to assess overall biocontrol potential carried out earlier in this project indicated an initial protective effect in T60-treated wood blocks buried in non-sterile soil, which subsided over time (section 2.3.1). The results of the field trial presented in this chapter support the findings of Tucker *et al.* referred to above, by indicating that the application of T60 spores for the bioprotection of wood in ground contact reduced the occurrence of basidiomycete decay in relation to untreated wood. In addition to this, soft rot indices presented in Table 4.9 showed that T60 pre-

treatment of wood resulted in a marked reduction in soft rot degradation. Although the applied biocontrol agent was not as effective as CCA in preventing the decay of wood in ground contact, partial protection against a range of soil biodeteriogens was observed in T60-treated stakes. Pre-treatment with *Trichoderma viride* isolate T60 had a significant effect on fungal colonisation and degradation of the test stakes, particularly by basidiomycetes, however, the environmental conditions test stakes were exposed to and wood species influenced the extent and nature of the protection observed.

Biological Control Agents

An important consideration in the research and application of biological control is that a biocontrol agent developed for the protection of wood must be shown to do so without adversely affecting the mechanical properties of the timber (primarily strength and elasticity). Other aspects influencing the acceptance of biological control agents such as *Trichoderma* spp. as viable alternatives to chemical preservatives include ease of application (i.e. application method and treatment solution); proven long-term protection; health and safety assurances; and the aesthetic appearance of the end product. Field trials designed to assess biocontrol agents under "in-service" conditions (i.e. relevant to the end use of the timber) provide a more comprehensive representation of how the biocontrol agent will perform in uncontrolled conditions and against a much wider range of potential target organisms, than tests assessing antagonism towards individual targets. As it is likely that the biocontrol agent will inhibit or kill some of the micro-organisms encountered but have no antagonistic activity against others, any subsequent effects of controlling one particular type of wood decay on microbial succession and the subsequent pattern of

colonisation of test specimens need to be investigated further. For instance, if pre-treatment of wood with T60 spores prevents basidiomycete colonisation of wood, will the absence of basidiomycete fungi have a subsequent effect on the rate of soft rot colonisation and degradation? Treatment of wood with CCA was shown by Clubbe and Levy (1982) to affect microbial succession by eliminating the establishment of basidiomycete fungi as the climax microflora, resulting in the substitution of soft rot fungi as the ultimate colonisers. Soft rot fungi require an elevated nitrogen level to colonise wood, therefore it must be ensured that the presence of the biocontrol agent does not encourage soft rot invasion in the course of protecting wood from basidiomycetes, for instance by increasing the nitrogen content or moisture content of the wood. Higher moisture contents were recorded T60-treated stakes and excessive changes in moisture availability are known to influence the type of colonisation observed (King, 1981; Figure 1.2, "Moisture ranges for the colonisation of wood by micro-organisms").

In this field trial, and in the accompanying fungal cellar test, no significant increases were observed in the soft rot indices of T60-treated stakes compared with untreated stakes, therefore establishing that the presence of the biocontrol agent T60 and its activity against basidiomycetes did not promote soft rot decay. Overall, no exacerbation of decay was observed in T60-treated wood over untreated wood during this study; despite the indications of increased surface softening associated with elevated moisture contents in T60-treated wood (Table 4.4), colonisation of test stakes by T60 spores did not appear to have any detrimental effects on wood soundness, based on the results of subjective analysis and pilodyn testing.

The efficacy and mechanisms of action utilised by *Trichoderma* spp. as a biological control agent will be influenced by a number of factors – the identity of the

target organism and strain of BCA selected are the most obvious factors governing the antagonistic activities and their effects. As primary colonisers such as moulds are dependent on the same non-structural nutrients present in the wood for growth and establishment as the pre-colonised *Trichoderma*, inhibition by the biocontrol agent will take the form of competition for nutrients. Basidiomycetes, however, are capable of degrading wood to obtain nutrients and as such, antagonism by *Trichoderma* is more likely to be by the production of volatile and soluble metabolites (Horvath *et al.*, 1995). However, environmental conditions and substrate (i.e. wood species) will also have a significant effect on the biocontrol capacity of potential isolates. A notable outcome of field and fungal cellar testing of *Trichoderma viride* T60 was the extent to which environmental factors influenced the performance of the biocontrol agent.

Laboratory studies have shown that temperature has a particular influence on whether antibiotic metabolites produced by *Trichoderma* spp. are soluble or volatile (Tronsmo and Dennis, 1978) and when *T. harzianum* metabolites were added to media used for basidiomycete interaction plates, temperature was found to influence the outcome of interactions (Schoeman *et al.*, 1996). *Trichoderma* spp. are known to be sensitive to abiotic environmental factors such as temperature and moisture (Hjeljord and Tronsmo, 1996), and it was clear from the observations made during this field trial that these aspects played a significant role in the efficacy of the applied biocontrol agent.

Laboratory investigations have also shown that media composition directly affects the modes of action of *Trichoderma* (Srinivasan, 1993) and other potential biocontrol agents (Benko and Highley, 1989). The production of antibiotic metabolites and siderophores by *Trichoderma* spp. was shown to be markedly

different when interactions were carried out on media with a carbon:nitrogen ratio similar to wood, compared with malt extract agar (Srinivasan, 1993), and more detailed examination of the production of volatile organic compounds (VOCs) by *Trichoderma* spp. evaluated the effect of subtle changes in amino acid composition of media on antagonism (Bruce *et al.*, 2000). Although Scots pine and Sitka spruce have similar nutrient compositions as substrates, it has been established that the nutrient status of pine is sufficiently higher than that of spruce for differences in fungal colonisation to be apparent (Breuil, 1998; Brown and Bruce, 1999). As even minor changes in the amino acid make-up of growth substrate have been shown to have a significant effect on VOC production by *Trichoderma* (Bruce *et al.*, 2000), it must therefore be considered that the more noted differences in nutrient composition between wood species will influence the behaviour and antagonism of potential biocontrol agents.

Nutrient availability, temperature, moisture and target organism are all factors affecting the mode of action of biocontrol agents, and it is logical that these factors will affect different biocontrol isolates in different ways. The altered antagonistic capacity observed in this field trial is an indication of the sensitivity of Trichoderma spp. to environmental factors. As relatively small changes in temperature and moisture availability affect spore production and spore germination, this will ultimately have an effect on the mode of action activated and the concentration of metabolites produced such as lytic enzymes, volatiles or soluble antibiotics. It is also worth noting that unlike the biocontrol of fungal plant pathogens where the substrate is effectively "live", wood in-service cannot interact with the applied biocontrol agent in order to exclude biodeteriogens.

Results reported in this chapter have demonstrated the antagonistic activity of the applied biological control agent, *Trichoderma viride* (isolate T60), evidenced by

the observed reduction of attack by wood decay fungi in T60-treated field and fungal cellar stakes. Although the level of protection noted was not as effective as that of the reference wood preservative CCA, T60-treated stakes nevertheless performed better than untreated control stakes in a number of different assessments of biocontrol potential. However, the most significant outcome of the field trial and fungal cellar tests was the observation that the environmental conditions a biological control agent is exposed to during testing are fundamental to its performance; in addition, the testing environment must be comparable to the intended service environment. It was observed that wood species and environmental conditions strongly influenced the colonisation, persistence and performance of T60 as a biocontrol isolate. The suitability of *Trichoderma* spp. as potential biocontrol agents is due in part to the ecological adaptability and abundance of this micro-organism (Hjeljord and Tronsmo, 1996). The principal conclusions drawn from the results presented in this chapter are that although partial protection of T60-treated wood placed in ground contact was achieved, further investigation is required into the effects of *Trichoderma* pre-treatment on permeability and moisture conduction in wood. In addition, the influence of environmental variables such as temperature and moisture availability on the antagonistic activity and long-term viability of an applied biocontrol agent observed in this study means that, in order to accurately assess the efficacy of potential biocontrol isolates, the intended geographical situation and end use of wood should be reflected in the design of any field testing undertaken.

Chapter 5

Development of a Molecular-Based Detection System

5.1 Introduction

The industrial-scale pressure impregnation of fungal spores into Scots pine and Sitka spruce stakes described in chapter 3 represents a novel approach to the delivery of biocontrol agents. Therefore, no information was available regarding the efficiency of this application method used in conjunction with fungal spores. In order to determine the distribution and relative abundance of spores following pressure treatment, a detection method was developed to identify *Trichoderma viride* (T60) using nucleic acids extracted from wood infected with T60 spores. A molecular-based method with the potential for specific detection and quantification of target DNA was investigated, acknowledging the diversity of such tools and making use of recent advances of this branch of science.

The development of molecular biology in the past 15-20 years has increased the variety of techniques available, and the range of these techniques applicable to environmental mycology has increased accordingly. For example, the polymerase chain reaction (PCR) has been used for the purposes of taxonomy, phylogeny and population studies (Edel, 1998). Molecular detection and identification methods such as enzyme-linked immunosorbent assay (ELISA) and protein profiling have previously been used to distinguish between fungal species (Palfreyman *et al.*, 1991; Burge *et al.*, 1994; Palfreyman and Vigrow, 1994). PCR has also been used for this purpose (Theodore *et al.*, 1995; Score, 1998; Dehal, 1999) and more specifically, in the distinction of different strains of the same fungal species (Zimand *et al.*, 1994; Arisan-Atac *et al.*, 1995; Edel, 1998; Schlick *et al.*, 1997)

Since the advent of PCR in the mid-1980s by Mullis *et al.* (1986), this molecular biology method has become a highly useful tool for biological researchers. The polymerase chain reaction allows selective amplification of a specific region of a DNA molecule (Brown, 1990). Amplification is achieved by repeated cycles of

denaturation, primer annealing and polymerase (enzyme) extension. In conventional PCR, the target DNA sequence or the sequence of the border regions of the target DNA must be known. Random amplification of polymorphic DNA by PCR (RAPD-PCR) does not require any prior knowledge of nucleotide sequence information, and utilises a single short, non-specific oligonucleotide sequence as a primer. The primer anneals to a number of sites on the template DNA, and will allow amplification of DNA fragments that are of undefined length and sequence (Edel, 1998). This means that, potentially, a RAPD PCR approach can produce a unique DNA pattern from the target DNA when PCR products are displayed on an agarose gel. However, the examples of specific detection of fungal species cited previously refer to detection achieved using either specific primers or template DNA derived from pure cultures of fungi, as opposed to the detection and identification of fungal DNA extracted from infected wood. Although Score (1998) reported the detection of *Serpula lacrymans* in timber through the extraction and amplification of DNA from infected wood using RAPD PCR, there are still difficulties associated with the extraction and subsequent PCR amplification of DNA from wood and plant tissues due to the presence of compounds such as tannins and polyphenols. (Brown *et al.,* 1993; Jasalavich *et al.*, 2000).

The work reported in this chapter was designed to develop and assess the application of a molecular-based detection system, based on PCR, in the field of biological control. This study considered the most appropriate approach for the development of a PCR-based detection strategy, taking into account the need to adapt existing methods to specific situations – in this case, the extraction of fungal DNA from treated or infected wood in order to detect a specific fungal isolate.

5.2 Materials and methods

The laboratory work undertaken for this chapter represents the development and assessment of a molecular-based approach to the detection of fungal spores in wood following pressure-impregnation with the selected biocontrol agent. As such, a number of protocols were investigated at several stages of the development process in order to optimise the procedures used.

Culture maintenance and media preparation are described in Appendix A: Culture Methods and Isolation. Chemical suppliers are detailed in Appendix B: Chemicals, Reagents and Suppliers.

5.2.1 Sample preparation

5.2.1.1 Pure culture preparation

Template DNA from pure cultures of *Trichoderma viride* (T60) was extracted and amplified for use as a positive control, i.e. as an indication of PCR reaction efficiency and as a reference 'fingerprint' to compare experimental samples against.

Plates of liquid nutrient media were inoculated with a 10mm core of T60 and incubated at 25°C for 7 days. Mycelial matter was removed from the plates, rinsed twice in sterile distilled water and blotted on sterile tissue before being placed in a sterile 1.5ml micro-centrifuge tube. The sample was centrifuged at 13,000 *g* for 2 minutes to remove excess water, before being used for DNA extraction as described in 5.2.2.

T60 cultures were also prepared by inoculating flasks of nutrient broth with spore suspensions (1 x 10^6 spores per ml) made from solid agar plates of T60 (as described in 2.2.1). The flasks were incubated for 3 days at room temperature and the mycelial material was then collected and prepared as above.

5.2.1.2 Wood sample preparation

Post-treatment wood samples

Five extra stakes of each wood species had been included in the treatment of stakes with T60 for use in the field trial (Ch. 3 & 4). These stakes were immediately cut into sections (see Figure 5.1) and the resulting sample blocks were milled using a Spex 6700 freezer mill (Glen Creston, Stanmore, Middlesex, UK). This reduced the samples to a fine powder, which was placed in sterile, labelled micro-centrifuge tubes and kept at –20 °C for storage. DNA was extracted from these samples using method D1 (5.2.2.1). These samples are referred to in this chapter as initial post-treatment wood samples.

Field trial samples

Wood samples were also retained from stakes uplifted during the course of the field trial and stored for later use, i.e. investigating the feasibility of extending the detection assay to include field and fungal cellar test stakes. Sections were cut from the stakes at the same positions shown in Figure 5.1. In the absence of the freezer mill used previously, the samples were removed from the cross-sections using a hand-drill to produce shavings from the pre-designated positions. The samples were collected in sterile, labelled micro-centrifuge tubes and frozen until required for DNA extraction using method D2 (5.2.2.2), and are referred to as field trial samples.

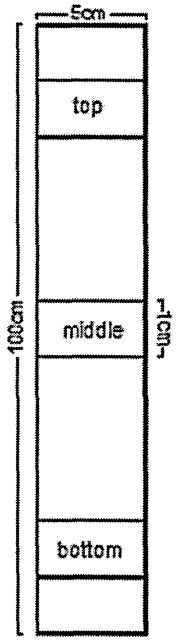


Figure 5.1 (a): Cross-sections were removed from stakes at 3 positions – top, middle and bottom.

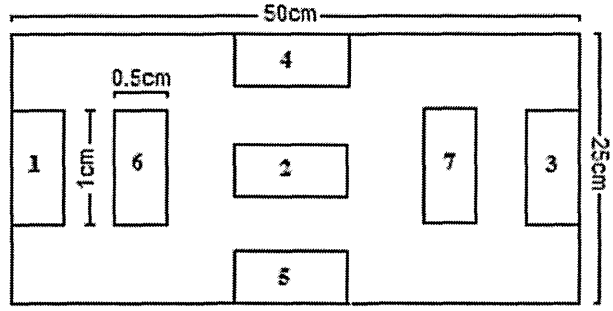


Figure 5.1 (b): Sampling sites within the cross-section

Figure 5.1: Positions of samples taken from stake cross-sections for PCR analysis.

Bench-top treated samples

The loss of all extracted DNA samples as the result of a freezer thaw before amplification and visualisation could be carried out led to the development of a smaller-scale replacement preparation. Blocks of Sitka spruce and Scots pine (measuring 100 mm longitudinal x 50 mm radial x 25 mm tangential) were pressure-impregnated with T60 spore solutions in a range of concentrations ($1x10^8$; $1x10^6$; $1x10^4$; 0 spores per ml) using the bench-top apparatus described in Chapter 3 (3.2.2.1). After the treated blocks had been dried, a 1cm-thick cross-section was removed from the mid-point (i.e. 45mm from either end) and using the same block template as for previous cross-sections (Figure 5.1), wood shavings were collected into sterile, labelled micro-centrifuge tubes for DNA extraction using methods D1 and D2 described in the following section. From excess test stakes (left in the fungal cellar after testing had essentially finished), 1 *Trichoderma*-treated stake of each wood species was prepared in the same manner as described for field trial stakes (previous paragraph).

5.2.2 DNA extraction

5.2.2.1 Method D1 (Score, 1998; adapted from Lee and Taylor, 1990)

Sterile labelled micro-centrifuge tubes were filled to the 0.5ml mark with frozen mycelium or finely-milled wood samples then dipped briefly in liquid nitrogen (LN_2). The samples were placed in a water bath at 65°C for 2 minutes and then vortexed. This process was repeated prior to the addition of 400µl of freshly prepared lysis buffer (600µl for wood samples to counter absorption of the solution by wood). Lysis buffer contained 1ml 30% sodium dodecyl sulphate (SDS); 0.1ml β-mercaptoethanol; 6.9ml sdH_2O; and 2ml [5x] Tris-EDTA (TE) buffer (50mM Tris-

HCl; 50mM diaminoethanetetra-acetic acid disodium salt (EDTA)). The samples were again vortexed then flash frozen in LN_2. The tubes were incubated in a water bath at 65°C for 1 hour, with flash freezing in LN_2 every 20 minutes during that time to disrupt the cell membranes. After incubation, 400 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the sample and briefly vortexed. The samples were then micro-centrifuged at 10,000 *g* for 15 minutes at room temperature. The aqueous phase (containing DNA) was removed to a fresh tube and an equal volume of chloroform:isoamyl alcohol (24:1) added. Samples were vortexed and the aqueous phase again transferred to a fresh tube. Ten microlitres of 3M sodium acetate was added to the aqueous phase material followed by the addition of 0.54 volumes of isopropanol. The tubes were gently inverted to mix the contents and then micro-centrifuged at 10,000 *g* for 2 minutes. The supernatant was decanted and the remaining DNA pellets were rinsed with 70% ethanol prior to re-centrifugation. The tubes were inverted for 1 minute and drained onto sterile paper towel. The DNA pellets were left to dry and then resuspended in 100 µl of sterile distilled water. The resuspended samples were then stored at 4 °C until used.

5.2.2.2 Method D2 (Dehal, 1999; adapted from Kolar *et al.,* 1988)

Frozen mycelium or chipped wood samples were placed in sterile labelled micro-centrifuge tubes to the 0.5ml mark prior to the addition of 400µl of lysis buffer (0.2M Tris-HCl; 0.25M NaCl; 0.05M EDTA (pH8.5) + 48mg/ml PAS (P-aminosalicyclic acid) and 8mg/ml TNS (Tris-*iso*-propyl-naphthalene-sulfonic acid sodium salt)). This solution was shaken well and allowed to settle on ice for 1 hour before use. A small amount of sterile Seesand was placed in each tube and the samples were ground using Treff homogenisers. The tubes were incubated for 20

minutes at 65 °C (dry heat, i.e. an oven or hot block, rather than a water bath) then allowed to cool to room temperature prior to incubation on ice for 20 minutes. Four hundred microlitres of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube, the samples were shaken gently and centrifuged at 10,000 *g* for 8 minutes. The aqueous phase was removed to a fresh sterile tube before the addition of an equal volume of chloroform:isoamyl alcohol (24:1). The samples were again shaken gently and centrifuged for 6 minutes, the aqueous phase was removed and this step was repeated. One tenth volume of ice-cold 3M sodium acetate (pH8) was added to each of the samples followed by 2 x volumes of ice-cold absolute ethanol. The tubes were gently inverted then placed at -20 °C overnight before centrifugation at 10,000 *g* for 10 minutes. The supernatants were discarded and the pellets rinsed in absolute ethanol, re-centrifuged, and the supernatant removed. The DNA pellets were allowed to dry, then resuspended in 100µl sterile distilled water and stored at -20 °C.

5.2.3 Assessment of DNA quality

The amount and purity of extracted DNA can be measured using ultraviolet absorbance spectrophotometry (Brown, 1990). A 1:100 dilution was prepared from extracted DNA. Absorbance was measured at wavelengths of 260nm and 280nm. An absorbance value of 1.0 at 260nm is considered equivalent to 50µg of double-stranded DNA. The ratio of the absorbance at 260nm against absorbance at 280nm (A_{260}:A_{280}) for pure DNA is 1.8. A lower absorbance value than this indicates contamination of the DNA by either proteins, phenols or both (Brown, 1990; Score, 1998). Extracted DNA samples were also electrophoresed on 1% agarose gels to visualise any DNA present. The preparation of agarose gels is described in section 5.2.6.

5.2.4 PCR set-up and optimisation

As part of the optimization process, basic parameters were established using pure culture T60 DNA before amplification of experimental samples commenced.

Reaction volume

Reaction tubes were set up containing both 25µl and 100µl reaction volumes to assess the most appropriate volume. A primer known to detect *Trichoderma* DNA was used (GGGACGTTGG – Score, pers. comm.).

Primer selection

A selection of primers from Operon Technologies RAPD primer kit D (suppliers' details can be seen in Appendix B) were tested to determine which primer would be appropriate for the amplification of T60 DNA, the sequences of which are as follows:

OPD-01	$^{5'}$ ACCGCGAAGG $^{3'}$
OPD-02	$^{5'}$ GGACCCAACC $^{3'}$
OPD-03	$^{5'}$ GTCGCCGTCA $^{3'}$
OPD-08	$^{5'}$ GTGTGCCCCA $^{3'}$

Once the basic PCR protocol had been optimised, a number of PCR amplifications were carried out, initially comparing T60 to 3 other *Trichoderma* isolates to establish the pattern produced by T60 with regard to that of other *Trichoderma* isolates using the same random primer; and comparing DNA extracted from T60 spores to that extracted from T60 mycelia. The purpose of this was to establish whether the strength of the DNA profile obtained from amplification of T60

was affected by the presence or absence of spores (fungal spores are known to be hardy structures and can be difficult to disrupt, reducing the yield of extracted material).

5.2.5 PCR amplification

5.2.5.1 Method A1

Optimization and initial PCR amplifications were carried out using the recipe and program presented in Tables 5.1 and 5.2 respectively. DNA extracted from one of the post-treatment spruce stakes using method D1 (5.2.2.1) was amplified in groups of 7 samples, corresponding to the blocks taken from each cross-section. In control samples where no template DNA was used, an equivalent amount of sterile distilled water was added, in order to keep reaction volumes consistent. Positive and negative amplification controls were included in every set of reactions, containing pure culture T60 DNA and sterile water respectively. These controls served as an indication of amplification efficiency and sterility.

Sterile micro-centrifuge tubes were labelled, and then the reagents listed in Table 5.1 were combined into a "master mix", before 95µl of the mix was added to each tube resulting in the volumes indicated. Template DNA was added to each tube as appropriate, and the contents of each tube was overlaid with 2 drops of sterile mineral oil for thermal insulation and also to act as a barrier to contaminants.

Reagent	Volume (µl) Master mix x10	Per tube
Reaction buffer♦ [10 x]	100.0	10.0
Taq DNA polymerase (1.1U)	10.0	1.0
dNTP's (100µM)	160.0	16.0
Primer (OPD-02)	80.0	8.0
Water (sdH_2O; later HPLC grade)	600.0	60.0
Template DNA (added to each tube individually)	(5.0)	5.0

♦ [1x] containing 16.6mM $(NH_4)_2SO_4$; 0.45% Triton X-100; 200mg/L gelatin in 67mM Tris-HCl, pH8.8

Table 5.1: PCR formula for amplification method A1

Amplification was carried out using a TECHNE PHC-3 thermocycler, using the programs listed in Table 5.2.

Program	Segment	Temp	Time		Purpose
P1	-	94 °C	4 min		denaturing
P2	A	94 °C	1 min	P2 x 45 cycles	denaturing
	B	36 °C	2 min		annealing
	C	72 °C	1 min		extension
P3	-	72 °C	10 min		extension
P4	-	6 °C	samples held until required		

Table 5.2: PCR programs for amplification method A1

5.2.5.2 Method A2

This method was used to amplify samples extracted from the bench-top treated blocks and pure culture T60 DNA which were prepared as replacement samples (section 5.2.1). Following a revision of the previous PCR protocol, reactions were set up with a smaller reaction volume (25 µl) and an improved reaction mix. This included the use of a pre-prepared master mix containing the polymerase enzyme and buffering compounds. Sterile micro-centrifuge tubes were labelled, and the listed reagents were added in the amounts specified in Table 5.3.

Reagent	**Volume (µl per tube)**
PCR Master Mix♦	12.5
Primer (OPD-02)	2.5
Water (HPLC grade)	5.0
Template DNA	5.0
♦ (containing 50 units/ml *Taq* DNA polymerase in reaction buffer (pH 8.5); 400µM each of dNTP's; 3mM $MgCl_2$)	

Table 5.3: PCR formula for amplification method A2

As before, tubes were overlaid with 2 drops of mineral oil before amplification using the following programs (Table 5.4).

Program	Segment	Temp	Time		Purpose
P1	-	94 °C	2 min		denaturing
P2	A	94 °C	10 sec	P2 x 40 cycles	denaturing
	B	36 °C	1 min		annealing
	C	72 °C	1 min		extension
P3	-	72 °C	10 min		extension
P4	-	6 °C	samples held until required		

Table 5.4: PCR programs for amplification method A2

5.2.6 PCR product visualisation

PCR products were visualised using gel electrophoresis in conjunction with ethidium bromide staining, and the gels were then viewed using an ultraviolet light source. Ethidium bromide is a compound that binds to nucleic acids, and fluoresces under UV light (Brown, 1990). It is also a potentially mutagenic compound and as such, appropriate safety guidelines were strictly adhered to during both staining and UV visualisation. Polaroid photographs were taken of gels when possible, to provide a record of results and for use in the analysis of the gel. As with the rest of the developmental work, more than one approach was utilised.

5.2.6.1 Method G1

Initially, agarose gels were prepared by dissolving 2.0% w/v agarose in Tris-borate-EDTA buffer (TBE - 2mM EDTA; 10mM Tris-boric acid). When the gels were set, they were placed in the running tank and covered with 500ml running buffer (TBE, as described). PCR product (8µl) was mixed with 2µl of loading dye (30%

(v/v) glycerol, 10mg bromophenol blue, distilled water to 10 ml) and loaded into individual wells. Molecular weight markers (500 bp ladder; Appendix B) were placed in the outermost wells. The gels were run for 1 hour at 100V, after which time they were removed from the electrophoresis tank and stained in ethidium bromide (50mg in 100ml TBE) for 30 minutes. Following staining, the gels were viewed using a UV transilluminator.

5.2.6.2 Method G2

Latterly, agarose gels were prepared as above (1.2% w/v agarose in TBE), however ethidium bromide (100ng/ml) was added to the agarose solution prior to pouring. PCR product (5µl) was mixed with 1µl of blue/orange loading dye (Promega) and heated at 65 °C for 2 minutes. The samples were then loaded into the wells, along with molecular weight markers pBr Taq I/Hae III mix, 400ng/5µl (see Appendix B). The gels were run at 137mA for 45 minutes, following which time they were removed and viewed as before, without the need for a staining step.

5.2.7 Gel analysis

Photographs of suitable gels were loaded into an image analysis program (Quantimet Q600, Leica Cambridge Ltd., Cambridge, UK and Microsoft) using a colour video camera (3CCD, Sony). The gel images were then transferred to Phoretix 1D Lite (Phoretix International Ltd., Newcastle-upon-Tyne, UK) and analysed by detecting peaks corresponding to bands in the DNA profile. The height of the peaks was related the intensity of the DNA band. As the image showed UV fluorescence of ethidium bromide-stained DNA, it was postulated that increased fluorescence (peak intensity) was related to the amount of DNA present in the analysed band.

5.3 Results

5.3.1 DNA extraction

Two methods were used to extract nucleic acids from both pure culture fungal material and wood which had been pressure-impregnated with T60 spores. The efficiency of extraction of DNA from pure fungal cultures was variable, both between techniques and within a single technique; however, it was the extraction of fungal DNA from treated or infected wood that presented the greatest difficulties. Using method D1 (5.2.2.1) with pure fungal culture material, the extracted DNA solution was generally of low concentration and relatively low purity (ranging between 50 - 70μg; 1.3 purity), whereas the DNA extracted from fungal cultures using method D2 (5.2.2.2) was a higher yield and better quality (around 400μg of DNA; 1.6 purity). Thus, method D2 appeared to be a more efficient DNA extraction procedure.

DNA extracted from the initial wood samples (those collected immediately following treatment with spores), using method D1, was of moderate yield (60-80μg; 1.2 purity); however DNA extracted from field trial and fungal cellar samples using method D2 was virtually undetectable. The samples from the bench-top treated wood were initially extracted using method D2, but the DNA yield was again undetectable (except as an indistinct smear on a gel, believed to be degraded DNA or RNA), with the exception of samples from stakes removed from the fungal cellar after around 29 months in direct soil contact (it was noted that the effects of decay resulted in the wood being much easier to grind up than sound wood, leading to better DNA extraction). The remaining starting material from the bench-top samples (in the form of wood shavings) was then extracted using method D1, which also proved unsuccessful.

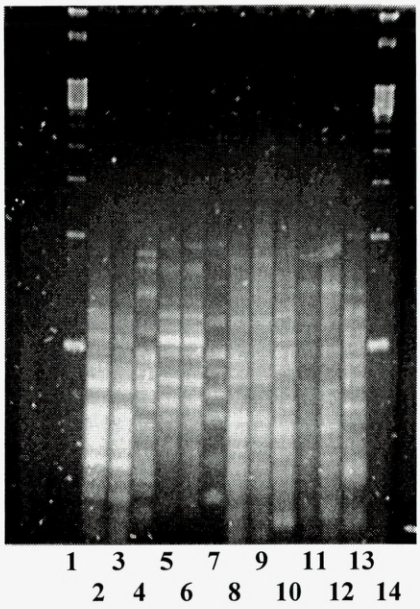
5.3.2 PCR set-up

Reaction Volume

Initially, as the PCR products from the 100µl reactions appeared to be of better quality, this reaction volume was used. However, later PCR reactions were carried out using the smaller reaction volume and shorter cycle lengths (5.2.5.2) after the system was re-optimised.

Primer Selection

The results of the primer evaluation process are shown in Figure 5.2. Primer OPD-02 was selected on the basis of a well-defined DNA pattern when samples were run on a gel (lanes 5 and 6), and was used in subsequent PCR experiments to detect T60. The incomplete optimization is also apparent in this gel, compared to later results (Figures 5.3 and 5.4). There is a high degree of smearing of the sample which generally occurs when the magnesium concentration in the reaction buffer is too high. The bands present in lanes 4, 7, 10 and 13 were initially thought be due to gross contamination of the PCR reagents, although the bands do not appear to be present in the same positions in the T60 lanes. It was later established that PCR conditions required further optimization to improve the stringency of amplification.



Lane 1	marker (500bp ladder)
Lane 2	OPD-01, T60
Lane 3	OPD-01, T60
Lane 4	OPD-01, control
Lane 5	OPD-02, T60
Lane 6	OPD-02, T60
Lane 7	OPD-02, control
Lane 8	OPD-03, T60
Lane 9	OPD-03, T60
Lane 10	OPD-03, control
Lane 11	OPD-08, T60
Lane 12	OPD-08, T60
Lane 13	OPD-08, control
Lane 14	marker (500bp ladder)

Figure 5.2: Agarose gel electrophoresis results of primer testing.

Testing of the selected primer against 3 other pure culture strains of *Trichoderma* demonstrated that a reasonable degree of distinction was provided by primer OPD-02, however this was to be expected as the samples being tested were single, pure culture DNA extracts and there was no ambiguity regarding origin of detected bands. Unfortunately, no photographs were obtained of these results.

Comparison of DNA extracted from segregated mycelial matter and spores of T60 was inconclusive, and although no obvious differences were observed, amplification was variable. Problems with contamination in the laboratory water supply, equipment and autoclaves utilised for the experiment resulted in a number of changes being made to working methods and sources of sterilisation. Changes made to the protocols in the process of optimisation and contamination control included the replacement of sterile distilled laboratory water with HPLC grade purified water; the use of pre-sterilised plastic-ware such as tips, micro-centrifuge tubes etc.; the use of either dedicated pipettors for use only with DNA or barrier tips, to reduce aerosol

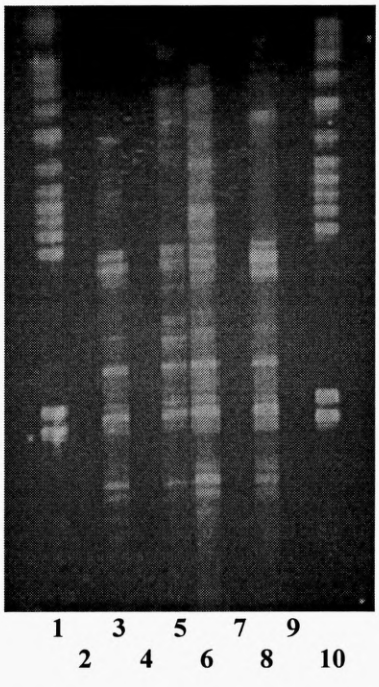
contamination; and the eventual replacement of some PCR reagents with a commercially available ready-made Master Mix (see Appendix B). This proved to be helpful in reducing both the contamination potential and the time taken to set up reactions as well as reducing the impact of pipetting errors, improving the overall efficiency of the PCR process.

The use of hot-start PCR was evaluated to determine whether this could improve the quality of the products obtained. Hot-start PCR is the term used to describe reactions where the *Taq* polymerase enzyme is added once samples have reached 94 °C and is used to minimise the polymerase activity that can occur at room temperature. Although reactions prepared in this way showed some improvement, with fewer spurious bands in control lanes, it was not adopted as a strategy due to practical considerations. A nested approach was also assessed, where a small aliquot (1-5µl) of PCR product is used as the template for a second round of PCR. This strategy is useful for strengthening weak DNA signals, however any contaminants present in the original reaction may also be amplified. The outcome of nested PCR carried out on T60 DNA during the optimization process was inconclusive, as no improvement in band quality could be ascertained, and contaminants present in the original negative control were exaggerated in the second round reaction.

5.3.3 PCR amplification and visualisation

Figure 5.3 presents the gel electrophoresis results of PCR amplifications carried out on post-treatment wood samples from a spruce stake following pressure-impregnation with T60 spores. The gel shows that fungal DNA, potentially that of T60, is present in 2 outer samples (lanes 5 and 8) and in 1 of the 2 intermediate samples (lane 6). It also shows that target DNA is not present in the sample taken from the centre of the cross-section (lane 7). The negative control (lane 2) is clear,

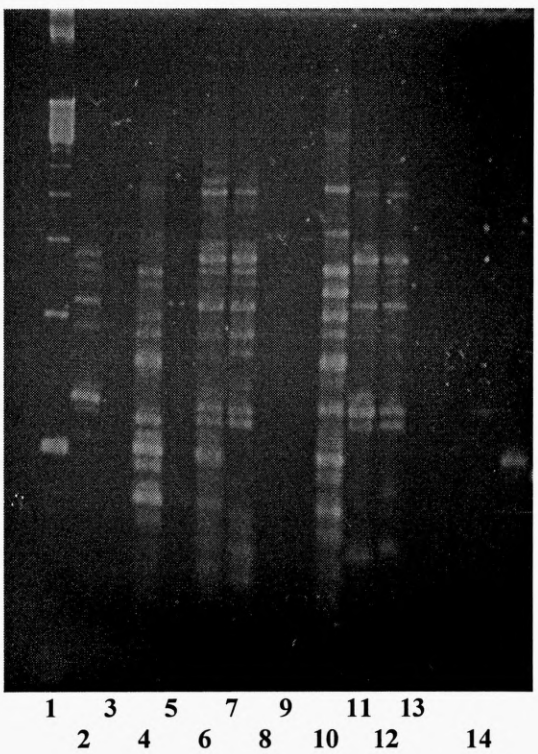
indicating the sterility and optimization of the reaction, and the T60 positive control lane has a well-defined banding pattern for comparison. Wood control samples did not amplify using the selected primer (lane 4), and so were not included as a control in future reactions. The banding pattern in the 2 outer samples (lanes 5 and 8) closely resemble that of the pure culture control, however, additional bands are present in the intermediate sample. As the negative control was clear on this occasion, the extra bands of amplified DNA may be from other organisms present in the wood. These results demonstrate that the presence of T60 is detectable in the wood samples following pressure impregnation, but also indicate that the spores are distributed in a shelling pattern, with no penetration to the centre samples.



Lane 1	molecular weight marker
Lane 2	-ve control (no DNA)
Lane 3	+ve control (T60)
Lane 4	spruce control
Lane 5	sample 1 (outside)
Lane 6	sample 6 (intermediate)
Lane 7	sample 2 (centre)
Lane 8	sample 4 (outside)
Lane 9	sample 7 (intermediate)
Lane 10	molecular weight marker

Figure 5.3: Agarose gel electrophoresis results of PCR carried out on DNA extracted from post-treatment wood samples. Molecular weight markers used - pBr Taq I/Hae III mix, 400ng/5µl

Figure 5.4 shows an agarose gel containing PCR products from fungal DNA extracted from cross-sections of decaying T60-treated spruce and pine stakes (recovered from the fungal cellar). The gel shows that the profiles in the sample lanes (4-12) are vastly different from the banding pattern in the T60 control (lane 2). The appearance of extra bands in RAPD PCR products indicates that the non-specific primer is amplifying other microbial DNA in addition to T60. In such instances, the origin of bands that correspond with the positive control lane cannot be ascertained. The results presented in Figure 5.4 illustrate the unsuitability of this RAPD PCR approach for detecting T60 from wood infected with such a wide range of organisms.



Lane 1	molecular weight marker
Lane 2	+ve control (T60)
Lane 3	-ve control (no DNA)
Lane 4	sample S G 1
Lane 5	sample S G 2
Lane 6	sample S B 1
Lane 7	sample S G 6
Lane 8	sample P G 6
Lane 9	sample P B 6
Lane 10	sample S B 1 (replicated)
Lane 11	sample P B 1
Lane 12	sample P G 2
Lane 13	empty
Lane 14	molecular weight marker

S = spruce **P** = pine
G = ground line **B** = bottom
1, **2** etc. = position in cross-section

Figure 5.4: Agarose gel electrophoresis results showing amplification of DNA samples extracted from spruce and pine, uplifted from fungal cellar. Molecular weight markers used - pBr Taq I/Hae III mix, 400ng/5µl

5.3.4 Gel analysis

Image analysis (Figure 5.5) was undertaken on the gel in Figure 5.3 indicates the presence of T60 spores in wood immediately following treatment. Relative quantification using the Phoretix gel analysis software gave an indication of the distribution of T60 spores in the wood sample, by using densitometry to analyse the intensity of DNA stained with a fluorescent dye. The height of the peaks representing a DNA band in the sample lane can be related to the intensity, and thus to the amount of DNA present in the band. However, concentration of DNA in a PCR product is not necessarily in direct proportion to the amount of DNA in the extracted sample, although it can provide an indication of distribution and relative signal strength. In this manner, it was determined that outer samples did not necessarily have a higher concentration of fungal DNA than samples from inside the wood. The absence of DNA detected in sample 2 (from the centre) suggests that pressure-impregnation of fungal spores is effective in applying spores into the wood, although not in an even distribution. In addition to this, image analysis of the gel also suggested that one particular surface sample had less DNA than the other outer sample and one of the intermediate depth samples, which may indicate that exposed wood surfaces in the treatment pile could be susceptible to a "washing" effect as the treatment liquid flows past, removing spores from the surface of the stake. However, it is equally possible that the reduced amplification was related to unavoidable handling of the sample during cutting and sample preparation.

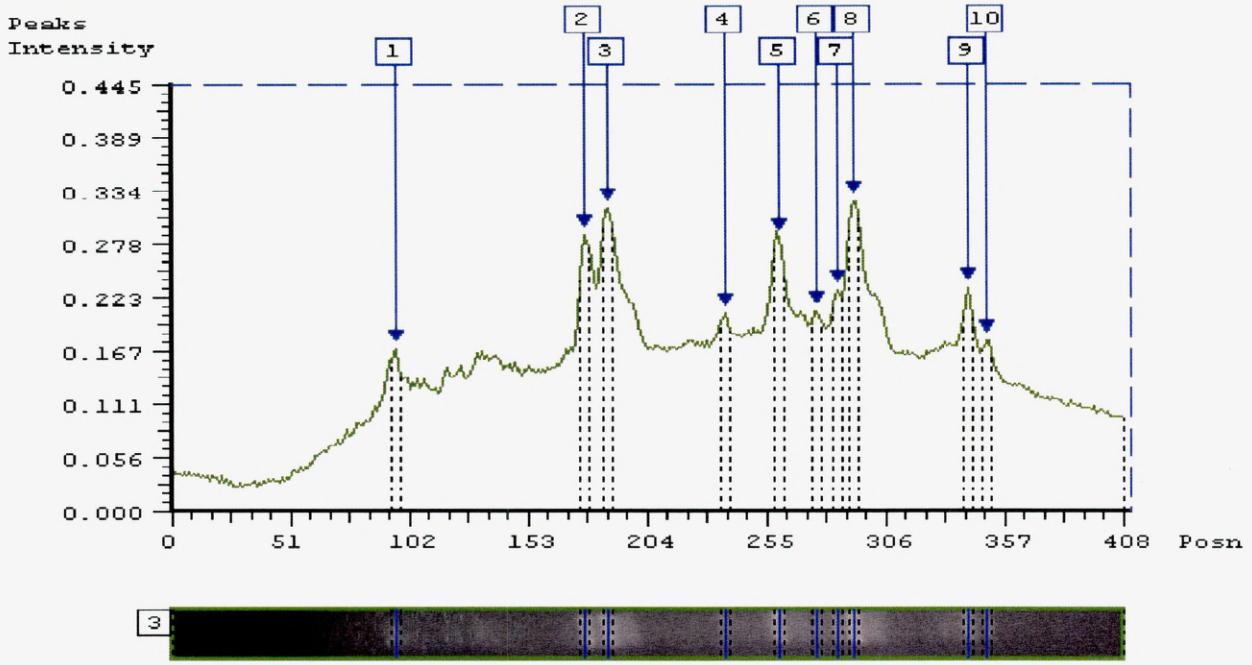


Figure 5.5: Data obtained from image analysis carried out on the agarose gel pictured in Figure 5.3

5.4 Discussion

The potential applications of a method for the specific detection and quantification of biomass are numerous, particularly for use in the assessment and monitoring of biological control agents. The polymerase chain reaction (PCR) allows the use of a standard method that can be adapted to almost any application. The results of preliminary investigations into the application of RAPD PCR to the detection and quantification of an applied biocontrol agent in wood were described in Brown and Bruce (1997), and the results presented in this chapter represent these investigations and further evaluation of the protocols used for the development of a molecular-based detection system for this purpose.

Unsuccessful DNA extraction in this project was attributed principally to the source and preparation of starting material (i.e. wood), and these factors could not be significantly altered although attempts were made. Extraction of fungal DNA from wood can be particularly problematic for a number of reasons, including the presence of naturally occurring phenols in plant material and decayed wood, which inhibit extraction and subsequent amplification (Jasalavich *et al.*, 2000; Scott *et al.*, 2000); low amounts of fungal DNA present in starting material in proportion to the wood itself; and the hardy nature of fungal spores which makes them difficult to lyse. Despite the inclusion of an extra chloroform:isoamyl alcohol step in both extraction methods (to maximise phenol removal), the presence of naturally occurring phenols and other organic compounds in the wood samples proved to be a particular problem, inhibiting extraction and subsequent amplification, and affecting the yield and purity of DNA obtained from wood samples.

Wood samples from the original stakes (taken immediately following industrial-scale pressure-impregnation with spores) were milled to a fine powder prior to DNA extraction using method D1, whereas field trial and bench-top treated

wood samples were obtained at a later date, using a hand-drill, and the fragments of wood were considerably larger than the initial powder-like samples. Due to the differences observed in particle sizes between preparation methods, the moderately successful extraction of DNA from a decaying stake from the fungal cellar was attributed to the observation that the decayed wood was easily crumbled. Although DNA extraction was attempted from these samples using the more effective method (i.e. method D2), the outcome of the extractions made it clear that the preparation of the starting material is of vital importance to the success of the subsequent extraction procedure. For most DNA extraction methods, protocols recommend that starting material is ground to a fine powder. This was not possible for the later wood samples, causing fundamental problems with the extraction process. This hypothesis is supported by results achieved for DNA extraction from pure culture T60 material, when amplifiable DNA was extracted from the ground up frozen mycelia using both extraction methods, and the extraction of DNA from milled wood samples only.

Removal of wood from blocks for DNA extraction has been reported using drilling (Jasalavich *et al.*, 2000), however the material obtained (using a cordless drill) was suitably powder-like, whereas the use of the hand-drill in this study resulted in coarse shavings and small chips of wood which could not be broken down further. Other authors reporting the successful extraction of DNA from wood and other environmental samples have utilised a variety of approaches to combat the effect of inhibitors on the procedure. Rigorous shaking of sample tubes containing material to be extracted and glass beads, i.e. bead-beating, has been used to macerate material that could not easily be broken down prior to lysis in an attempt to increase yield (Steffan and Atlas, 1991). Protocols incorporating bead-beating and cetyltrimethylammonium bromide (CTAB) buffers have greatly improved DNA extraction from environmental samples such as soils (Griffiths *et al.*, 2000), and

Jasalavich *et al.* (2000) reported that the use of CTAB in the presence of β-mercaptoethanol resulted in successful extraction fungal DNA from inoculated wood. However, it was also noted that CTAB extraction buffers required dilution (1:2) prior to addition to wood samples, as the aqueous and organic phases of the extraction inverted due to rehydration of the wood when the standard concentration of buffer was used (Jasalavich *et al.*, 2000). Although CTAB was not used in this study, the addition of excess lysis buffer to wood samples to combat absorption of the lysis solution (method D1), may have resulted in inhibition of DNA extraction or amplification if lysing agents were present in too high a concentration. This may also have caused a similar inversion effect to that noted by other authors, and may have contributed to the poor quality of DNA isolated in this project.

DNA extracted from environmental samples often has a high proportion of organic compounds that can degrade DNA and inhibit amplification, and although extracted material can be cleaned by carrying out agarose gel or phenol:chloroform purification steps, this is time-consuming and can cause damage the DNA sample or increase other types of contamination. Suppliers of molecular biology equipment and reagents have addressed this difficulty, and in addition to DNA extraction kits which provide pre-prepared solutions, there are now commercially available DNA purification kits specifically designed to remove unwanted proteins and organic compounds with a minimum of damage to sample.

The purpose of this study was to evaluate the use of RAPD PCR for the detection of a specific *Trichoderma* isolate in inoculated wood. Random amplification of pure DNA generates multiple bands of DNA (DNA "fingerprints"), distinctive banding patterns that can be associated with a specific organism (Bridge and Arora, 1998). A number of authors have reported the successful use of RAPD PCR to compare and characterise fungal species, and to distinguish between different

strains of the same species (Zimand *et al.*, 1994; Arisan-Atac *et al.*, 1995; Theodore *et al.*, 1995). However, Mills *et al.* (1992) suggested that at least 2 sets of primers should be sued for RAPD analysis of fungal isolates, and Schmidt and Moreth (1997) referred to conclusions by Hering and Nirenberg (1995) that at least 4 RAPD primers are required in order to avoid spurious amplification results when performing RAPD PCR phylogenetic comparisons of *Serpula lacrymans*. However, the majority of research of this nature has been carried out on pure cultures of the fungi under study as opposed to environmental samples.

In this study, using non-specific RAPD PCR primers, wood-extracted DNA was amplified and compared to DNA extracted from pure cultures of T60. However, the amplification of DNA extracted from environmental samples proved to be more problematic than anticipated and the system required repeated optimization. It has been widely reported that substances present in plant material such as polysaccharides, phenolic compounds and humic acid are inhibitory to *Taq* polymerase activity (Scott *et al*, 2000), and some chemicals used in the extraction of DNA have also been found to affect amplification (Pastrik and Maiss, 2000). Consequently, it appears that DNA extracted from environmental samples in particular, requires careful but efficient purification in order to improve the quality of the material prior to PCR amplification.

Optimization of the PCR protocols used in this study included selecting an appropriate primer and assessing the contribution of hot-start and nested PCR approaches to the study. Spurious bands present in negative control samples suggested that PCR conditions required further optimization to improve the stringency of amplification, although one of the inherent characteristics of random amplification is the low stringency conditions of the reaction. After a number of changes to the protocol to eliminate contamination as a source of extra bands, hot-

start PCR was carried out (Perkin-Elmer, 1993). PCR reagents (except *Taq*) are added to tubes and a wax bead is melted onto the surface. The polymerase enzyme is added above the wax, ensuring that the enzyme cannot react with the other constituents of the reaction mix until the tubes are heated at the beginning of the amplification process and greatly reducing unwanted amplification. In the absence of wax beads, *Taq* can be added to tubes that have been pre-heated and held (for a short time) at the denaturation temperature, taking care to add the enzyme below the mineral oil cap. However, this increases the opportunity for contaminants to enter the reaction tubes, particularly when the thermocycler is situated in a communal laboratory area, and depending on the number of samples being amplified in each set, can be impractical and lead to failure of the reaction if tubes are held at high temperatures for too long. Therefore, although hot-start PCR may have helped to improve the stringency of the process, the increased risk of contamination and invalidation of samples meant that the approach was not used in this study.

Nested PCR, where PCR products are re-amplified, can act to increase poor yield of PCR products if the same primers are used, as reported by Jasalavich *et al.* (2000). However, different primers can be used for the second round of PCR but only if non-RAPD PCR is being used. The nested primer set binds within the region of DNA amplified in the first round, increasing the specificity and amplification efficiency by minimising non-specific annealing of the primer (Steffan and Atlas, 1991). Other means of improving amplification of environmental samples include the addition of bovine serum albumin (BSA), a compound that can "protect" DNA from PCR inhibitors resulting in increased product (Comey *et al.*, 1994; Jasalavich *et al.*, 2000).

The PCR experiments undertaken here demonstrated that although RAPD PCR was suitable for the detection of T60 spores in wood immediately following

treatment (Figure 5.3), this approach was not suitable as a detection method when the wood had been exposed to a wide variety of micro-organisms (Figure 5.4). Similar observations have been made by other researchers investigating the extraction and amplification of fungal DNA from wood (Jasalavich *et al.*, 2000), although the approach used was taxon-specific amplification of fungal DNA using primers targeting the internal transcribed spacer regions (ITS) to avoid amplification of plant (i.e. wood) DNA.

In addition to demonstrating the importance of sample preparation (i.e. particle size), the amplification of material extracted from decaying wood samples also illustrated that the ability (and indeed intention) of RAPD primers to amplify non-specific DNA results in additional bands in DNA profiles generated from wood exposed to a range of micro-organisms. In these instances, the origin of the detected DNA cannot be ascribed with any certainty, and in fact a single band of DNA visualised on an agarose gel is comprised of many different fragments of the same size but potentially different sequences. Therefore the use of a specific primer would make detection of a single micro-organism in environmental samples more accurate and this suggests that a *Trichoderma*-specific primer would need to be designed if the system was to be expanded to detect T60 in field trial samples.

Another important consideration in design of PCR experiments is the intended post-amplification analysis of the samples. There are a number of analyses that could be carried out to distinguish T60 DNA from the DNA of other *Trichoderma* spp. that would be amplified by a species-specific primer. However few, if any, of the available methods would be compatible with RAPD PCR. For example, denaturing gradient gel electrophoresis (DGGE), a technique used to separate DNA fragments based on single base pair differences in sequence (Sheffield *et al.*, 1990), requires the generation of same size fragments and the use of GC-clamped primers. Restriction

fragment length polymorphism (RFLP) of PCR products also provides fingerprint-style information but again, requires uniform fragment length prior to enzymatic digestion.

PCR products electrophoresed on agarose gels were analysed using Phoretix 1D Lite gel analysis software. The program detects the presence of bands on a gel and generates a profile for each lane, as shown in Figure 5.5. The height of the peaks representing a DNA band in the sample lane can be related to the intensity and thus the amount of DNA present in the band. DNA on agarose gels can be quantified using densitometry and comparison to known amounts of DNA (i.e. molecular markers loaded at a pre-determined concentration). It must be noted here, however, that this is a measure of the amount of PCR product not concentration of the original DNA extract, and that the concentration of DNA in a PCR product is not necessarily in direct proportion to the amount of DNA in the extracted sample. Too high a concentration of template can inhibit PCR to the extent where no product is generated and due to the "plateau" effect encountered during PCR (Morrison & Gannon, 1994), when saturation of the amplification occurs and amplification is no longer exponential, accurate quantification of DNA would require the use of techniques such as competitive PCR, where an internal standard is utilised, or real time PCR (Edel, 1998; Bates *et al.*, 2001).

The data generated by gel analysis can, however, be used to assess the relative abundance of DNA in PCR products performed on undiluted extracts from wood treated with T60 spores, although RAPD amplification will result in DNA of differing origins appearing as the same band on agarose gels.

In this preliminary assessment of gel analysis to quantify an applied biocontrol agent, it was determined that external samples did not necessarily have a higher concentration of fungal DNA than samples from inside the treated stake,

although this may have been the result of post-treatment handling of the stakes and cross-sections (i.e. removal of spores by rubbing, etc. which gloves do not prevent).

As not all the DNA samples extracted could be used in PCR experiments, due to either poor quality template material or loss of the extracted samples, the intended gel analysis was not completed and so definitive conclusions could not be drawn as to the suitability or efficiency of this approach to DNA quantification.

PCR was selected as the most appropriate approach to the development of this detection system, because of its potential for specificity, sensitivity and multiple applications, but also to maximise the potential of new technologies by applying them to more areas of biological research. Other fungal detection methods that were considered included ergosterol analysis, chitinase analysis and ELISA, which were thought to be less applicable than PCR for reasons including non-specific and non-quantifiable detection, or isolation plating, which requires familiarity with morphological identification.

There are also drawbacks to PCR, in that it is expensive to equip a laboratory to such an exclusive use. It is also time-consuming and requires optimisation for accurate results. The sensitivity of the method which is such an incentive can also be a problem, as strict precautions must be taken to successfully eliminate all potential contaminants, such as the use of segregated space and dedicated equipment (Orrego, 1990), which is not always available. Many molecular biologists assert that separate pipettors must be used for extraction, PCR set-up and PCR products in order to avoid contamination. Contamination remained a sporadic and unpredictable problem throughout the course of this work despite as many precautions as possible being undertaken (e.g. UV sterilisation of pipettors; gloves worn at all times and changed frequently; preparation of reactions in Laminar air-flow cabinets; aliquotted reagents; barrier tips etc.). Many were unfeasible due to either cost or availability of laboratory

facilities, however, personal experience has shown that in the appropriate laboratory conditions, amplification reactions can be on the bench with a minimal risk of contamination.

An important stage in PCR is the extraction and preparation of the template DNA. There are many protocols for these stages, some allowing the isolation of pure genomic DNA required for detailed molecular analysis. However, the amplification of fungal DNA from mixed DNA extracted from complex environments such as soil or plant materials, rather than pure cultures, is better achieved by the use of specific primers (Edel, 1998).

As reported in Brown and Bruce (1997), initial developmental work demonstrated the potential applicability of the proposed approach to a molecular-based detection system, however, continued appraisal of the methods used indicated that further development and evaluation would be more effective if directed at specific amplification of T60 using specially designed primer pairs and more complex analysis of PCR products. It was also considered that validation and application of a gel analysis system to quantify T60 in wood would be more pertinent to the proposed use if they were carried out on specifically-detected material. Other stages of the process also required intensive revision, including optimisation of sample preparation and overcoming problems associated with the extraction and amplification of genetic material from wood (or the utilisation of purification protocols if this cannot be achieved). In addition, extension of the detection system to monitor the presence of T60 for the duration of its use in the field would certainly require the generation of T60-specific primers or the utilisation of post-PCR analyses designed for the distinction of DNA sequences for specific detection, and the use of competitive or real time PCR followed by gel analysis would be necessary for accurate quantification.

Chapter 6

General Discussion

Wood in ground contact is at risk from attack from a range of biodeteriogens, including wood decay fungi. At present, chemical preservatives are used to prevent biodegradation of wood in service, however there is now increased concern regarding the toxicity of these formulations and their effect on the surrounding environment. The use of biological control to protect wood in ground contact from wood decay fungi is being investigated in response to the need for alternatives to chemical preservatives. Biological control utilises the natural antagonistic characteristics of a number of fungi, including *Trichoderma* spp. There are factors particular to the biological control of wood for use in service which make the development of a system a complex issue, such as the need for broad-spectrum protection by a single isolate for a service period of 20 years or longer.

The first aim of the project was to develop a small-scale wood-based screening system for the rapid laboratory assessment of the biocontrol potential of a number of *Trichoderma* isolates. The use of wood wafers and individual microcosms (i.e. bottles containing a single wood block) resulted in measurable weight loss from wood samples after just 3 weeks. A system which can demonstrate weight losses after a short test period will allow more rapid screening of high numbers of potential biocontrol isolates. The test system was developed using *Trichoderma viride* isolate T60, a known antagonist of several basidiomycete wood decay fungi. When tested against both non-sterile soil and sterile soil inoculated with a soft rot fungus (*Chaetomium globosum*), the results indicated an initial reduction in weight loss in T60-treated blocks compared with untreated controls in non-sterile soil, although there was no effect on blocks incubated in *Chaetomium*-infected soil. Therefore, the development of a laboratory-based screening system using wood wafers and non-sterile soil provided a rapid, reproducible method for evaluating the antagonistic

capacity of a number of potential biocontrol agents and their suitability for use with wood in ground contact.

The selection and use of non-sterile soil as a test medium allows screening of potential biocontrol isolates for broad-spectrum protective effects, which is more appropriate for ground contact timber-related studies than testing against single target micro-organisms. Target populations are an important factor influencing the mode of action and efficacy of a biocontrol agent and screening systems using non-sterile soil are a necessary precursor to field testing of biological control in ground contact timber, and are a more relevant indicator of performance in further tests.

The laboratory-based screening system could be improved by increasing the number of replicates used, reducing the effects of variability and increasing reproducibility; investigation into the mode of application of isolates to be tested would evaluate whether bench-top pressure impregnation would be more appropriate than dipping; and valuable information could be obtained by increasing the frequency of block uplifts where initial protection is indicated.

The non-sterile soil system was then used to screen a selection of potential biocontrol agents. A further 10 *Trichoderma* isolates were assessed for their protective effect against non-sterile soil, although none of the isolates tested demonstrated absolute control of decay. However, the weight loss values recorded validated the design of the test system in that measurable decay was observed in wood wafers exposed to non-sterile soil for 3 weeks. The screening system was also modified slightly to assess the biocontrol potential of a bacterial isolate, on the basis of observed inhibition of mould fungi in an interaction study. Although screening of the bacterial isolate was inconclusive, it did demonstrate the flexibility and adaptability of the screening system.

Although none of the isolates screened in the initial microcosm tests prevented the occurrence of decay, *Trichoderma viride* isolate T60 was selected as a potential biological control agent for use in a field trial experiment. This isolate, already known to be an antagonist of several basidiomycete fungi, exhibited an initial protective effect in wood blocks when screened using the non-sterile soil system (Brown *et al.*, 1996), and was also reported by Tucker *et al.* (1997) to be totally effective in controlling decay by selected brown and white rot basidiomycete fungi. The apparent transience of the control observed during screening suggests utilisation of a passive mechanism of control by the applied isolate, such as nutrient competition, slowing the establishment of soil organisms.

As only 10 *Trichoderma* isolates and one bacterial isolate were screened during this study, the developed system could be used to assess further potential biocontrol agents. In particular, isolates from genera other than *Trichoderma* should be tested, to determine whether other organisms have a broad enough target specificity for use as bioprotectants of wood in ground contact situations.

In order to set up a field test of the selected biocontrol agent, an appropriate delivery system for the application of fungal spores to wooden stakes was required. Tucker *et al* (1996) established that *Trichoderma* spores could survive bench-top pressure impregnation, therefore a delivery strategy was developed using vacuum pressure impregnation to apply fungal spores. The biocontrol agent was applied to field trial stakes as a spore suspension prepared in sterilised water, using commercial pressure treatment regimes recommended for the treatment of pine. A pilot preservation plant, designed for the application of chemical wood preservatives, was used to treat wood with the biocontrol agent. Application and establishment of biocontrol agents in timber are crucial factors in determining the performance of

biocontrol of wood decay, and wood intended for use in ground contact must be treated in accordance with the appropriate guidelines. Germination of spores on T60-treated wood following incubation demonstrated the successful application of a fungal biocontrol agent using industrial equipment. Therefore, it was concluded that a biological control agent could successfully be applied using standard industry procedures and retain viability. Pressure impregnation of wood using a water-based solution ensured adequate inoculum, and moisture was better distributed throughout the substrate than that achieved using other methods such as painting, spraying or single-site inoculation.

Further investigation into the delivery of biological control agents using standard industry processes, including large-scale spore production and the use of selective inocula-directed nutrient solutions for application would be beneficial. A suitable method is required for the cultivation of large amounts of pure cultures of any proposed biocontrol agent. A system providing optimal nutrient, aeration and light conditions would reduce production time, and easy separation of spores from the growth medium and other fungal fragments also needs to be investigated.

The fourth aim of the project was to develop a field trial to test the selected biological control agent in an environment more closely resembling the proposed end use of preserved timber. European standard guidelines were used as a framework for the design of the field trial, in order to further demonstrate the applicability of standard industry practices to biocontrol strategies. The T60-treated stakes were planted in a field site, along with CCA-treated and untreated controls. Monitoring and laboratory assessment of the field trial stakes indicated a reduction in some types of fungal attack in T60-treated wood. The field trial results were similar to those of laboratory tests carried out by Tucker *et al.* (1997) in that T60-treated wood was more

protected against basidiomycete decay than untreated wood. *Trichoderma*-treated pine stakes demonstrated a reduced rate of sapstain discoloration in comparison with untreated pine controls, although it did appear that some T60-treated spruce developed sapstain at a slightly higher rate than untreated spruce. This indicates the effect of wood species on the behaviour and performance of the biological control agent. The observations and analyses carried out on field trial stakes also provided interesting results regarding the effect of *Trichoderma* treatment on wood moisture content, possibly due to enhanced permeability, as T60-treated wood was found to have much higher moisture contents (measured by dry weight analysis) than untreated wood. Overall, a degree of protection was observed in stakes pre-treated with *Trichoderma* spores, and there was no evidence that T60-treatment of wood resulted in increased decay compared to untreated wood.

The next aim was developed to supplement field trial testing of the selected biocontrol isolate. Stakes were planted in soil beds in a controlled environment with increased temperature and relative humidity (referred to as fungal cellar testing), to provide test conditions conducive to decay. The results of the fungal cellar testing gave a clear demonstration that stakes were exposed to more aggressive decay conditions in the controlled environment than field stakes. Decay assessment results showed that T60 had an obvious protective effect on spruce stakes in the fungal cellar, and this effect was more pronounced in the later uplift. As with T60-treated wood tested in the field, *Trichoderma*-treated cellar stakes showed consistently elevated moisture contents, despite tub-to-tub variations in moisture content. Again, this does not appear to have been due to extensive decay of any kind. Overall the fungal cellar results, like those from the field trial, illustrated that differences in wood species and environmental conditions such as temperature and moisture availability

can have a significant effect on the performance of a biological control agent. The results of fungal cellar testing of *Trichoderma viride* isolate T60 demonstrated the need for further investigation into the effects of variables such as testing and service environments and wood species on biocontrol potential.

Analysis of field trial and fungal cellar testing gave a more detailed indication of the biocontrol potential of the selected isolate. Reduced decay in T60-treated field stakes indicated a degree of protection from wood-degrading organisms, particularly in pine, however, T60 pre-treatment inferred more protection in spruce than in pine in the fungal cellar and this was more evident after 18 months. These patterns are reflected in the results of *Trichoderma* isolation from test stakes; seasonal effects on *Trichoderma* growth were indicated by fluctuating rates of isolation from field stakes, while more sustained and consistent isolation rates were obtained from cellar stakes. A similar pattern was observed with regard to protective effect: the lower frequency of *Trichoderma* isolation from field stakes after 18 months compared with that after 9 months is consistent with less obvious indications of protection over time. Comparison of field and cellar results concludes that environmental factors are the most influential factors in this observation.

Different environmental conditions, especially temperature and moisture availability, have an effect on the antagonistic properties of *Trichoderma,* including which specific mechanism of action or combination of mechanisms is likely to be utilised. This is also indicated when results from the field trial and fungal cellar are compared. T60 pre-treatment was also found to reduce the rate of sapstain colonisation in pine stakes, indicating its potential as a short-term protective agent. Overall, the results highlight the importance of field testing as a performance indicator for biological control agents.

Information regarding the long-term survival and antagonistic capacity of biological control agents still requires investigation. The field trial timeframe was devised with the time constraints of a Ph.D project in mind and this resulted in a limited period of testing. As the field trial was shorter than the recommended minimum period of 5 years, it is clear that a longer exposure period would be required for a more comprehensive assessment of biocontrol agent establishment and survival, particularly with regard to seasonal/climatic effects on growth, as would the use of more than one field site. Long-term survival of biocontrol agents also depends on nutrient availability, and whether antagonistic properties would be retained and exhibited over time. In addition, identification of key organisms in each test system may allow further investigation into specific and complemented modes of action by biocontrol agents.

The final aim of the project was the development of a molecular biology-based detection system, to assess the distribution of *Trichoderma viride* spores in wood following pressure impregnation. Initially a preliminary system using random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) and gel analysis software was developed. Using this system, T60 was detected in a number of samples taken from a cross-section of pre-treated wood, and analysis of T60 detected in these samples indicated a non-uniform distribution of spores in wood. The results of RAPD-PCR of DNA extracted from wood uplifted from the fungal cellar however, showed that RAPD primers could not specifically differentiate T60 DNA from other DNA present.

The molecular detection system developed for the detection of T60 spores in treated wood would require a substantial degree of manipulation in order to be applied to detection of T60 in soil-exposed wood. Practical issues with regard to

sample preparation and DNA extraction would first need to be refined to allow the extraction of clean nucleic material from wood samples; a more specific amplification process would also be required, using specific primers to reduce amplification of non-T60 material rather than the non-specific random primers used for RAPD-PCR analysis. Furthermore, in order for subsequent software-based quantification of amplified DNA to be accurate and achievable, a modified PCR method such as competitive PCR or real-time PCR would be required, due to limitations within the RAPD system.

Difficulties encountered with regard to the extraction of DNA from wood would need to be resolved for further investigation to be practical. These included the inhibition of extraction and PCR amplification by naturally occurring organic compounds in wood and plant tissues. In particular, the experiment highlighted the importance of sample preparation to the subsequent success of DNA extraction. Although gel analysis of one PCR gel gave a relative quantitative value to the amount of DNA present in sample lanes, due to experimental limitations actual quantification of PCR products in terms of the amount of T60 DNA in wood would require the use of alternative quantification methods such as real-time PCR, competitive PCR or isotope labelling. In addition, the detection of *Trichoderma viride* isolate T60 in wood exposed to a range of biodeteriogens (i.e. field and cellar samples), would require a species-specific primer and more in-depth analysis of PCR products to distinguish the target isolate from other organisms.

Overall, the project results indicate that the biocontrol isolate used, *Trichoderma viride* T60, exhibited a range of protective and antagonistic activities, although pre-treatment with a biological control agent did not completely prevent decay occurring in ground contact timbers. A successful delivery system was

developed, however long-term survival of biocontrol agents needs to be established under field conditions reflecting the end-use of such timbers. The effects of environmental factors such as temperature, moisture, target pool and nutrient availability need to be more fully assessed with regard to their influence on mode of action and subsequent efficacy, providing a comprehensive view of the potential application of biological control for the protection of ground contact timbers.

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Appendix A: Maintenance of Stock Isolates and Selective Media

The soft rot isolates used, as outlined in European Standard ENV 807 "Wood preservatives - Determination of the toxic effectiveness against soft rotting micro-fungi and other soil inhabiting micro-organisms" (CEN, 2001), were:
Chaetomium globosum Kunze (ATCC 6205); *Humicola grisea* Traen (MG 28); *Trichurus spiralis* Hasselbr. (MG 31); *Petriella setifera* (Alf. Schmidt) Curzi (MG 50); *Phialophora mutabilis* (van Beyma) W. Gams and McGinnis (S 24-E). All soft rot cultures were maintained on 5% malt extract (Oxoid L39) / 2% agar (Oxoid L28) at 22°C and sub-cultured regularly.

The *Trichoderma* isolates used in this study were:
Trichoderma viride (Persoon ex S. F. Gray) isolates T14, T28, T40, T53, T60, T70, T100, T110 and IMI 49791 and *T. pseudokoningii* (Rifai) isolates T51 and T64. All *Trichoderma* cultures were maintained on 5% malt extract agar (Oxoid CM59) at 25°C and sub-cultured regularly.

A bacterial isolate found inhibiting mould fungi on a laboratory petri dish was sub-cultured onto 3% malt extract agar (MEA; Oxoid CM59), and tentatively identified as a strain of *Pseudomonas* spp. The isolate was maintained on 3% nutrient agar (Oxoid CM3) at 25°C and sub-cultured regularly. Liquid cultures of the *Pseudomonas* isolate were prepared using yeast peptone dextrose (YPD) broth as described by Benko (1989), for use in the screening system.

In order to obtain pure culture fungal material for DNA extraction and amplification, plates of liquid nutrient media were prepared using nutrient broth (3%; Oxoid CM01). These were then inoculated with a 10mm core of T60 from a solid agar plate, and incubated at 25°C for 7 days. Liquid cultures of T60 were also prepared by inoculating flasks of nutrient broth (3%; Oxoid CM01) with spore suspensions (1 x 10^6 spores per ml). Flasks were incubated at 25°C for 3 days, prior to removal of mycelial matter for DNA extraction.

Low Nutrient Media (Huttermann & Volger, 1973)

Constituent	**Amount per 1.0 L dH_2O**
D-glucose	5.000g
Potassium dihydrogen orthophosphate (KH_2PO_4)	1.000g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.614g
Potassium chloride (KCl)	0.500g
Iron sulphate ($FeSO_4.7H_2O$)	0.010g
Manganese acetate ($Mn(CH_3COO)_2.4H_2O$)	0.008g
Zinc nitrate ($Zn(NO_3)_2.6H_2O$)	0.002g
Calcium nitrate ($Ca(NO_3)_2.4H_2O$)	0.050g
Copper sulphate ($Cu\ SO_4$)	0.002g
Ammonium nitrate (NH_4NO_3)	0.008g
Purified agar	10.00g
added after autoclaving:	
L-asparagine	0.013g

Basidiomycete Isolation (Clubbe and Levy, 1977)

Constituent	**Amount per 1.0 L dH_2O**
Malt Extract Agar	30.00g
Benomyl* (8μg/ml stock solution)	1.000ml
added after autoclaving:	
Streptomycin sulphate	1.000g

*** Benomyl stock solution**

Benomyl (1-butyl carbonyl-2-benzimadazole carbamic acid methyl ester (Hale and Savory, 1976) is a heat-stable wettable powder; benomyl in powder form (Benlate) comprises 50% "filling agent" and as such, calculation of stock solution concentrations must take this into account. A double-strength solution was prepared by dissolving 0.8 μg Benlate in 100ml of 50% ethanol. To obtain a final concentration of 4ppm, 1ml of the stock solution was added to 1L of malt extract agar.

Basidiomycete Differentiation (Nobles, 1948)

Constituent	**Amount per 1.0 L dH_2O**
Malt Extract	15.00g
Purified agar	20.00g
added after autoclaving:	
Gallic acid	5.000g

Trichoderma Selective Media (Elad *et al.*, 1981)

Constituent	**Amount per 1.0 L dH_2O**
Magnesium sulphate ($MgSO_4.7H_2O$)	0.200g
Dipotassium hydrogen orthophosphate (K_2HPO_4)	0.900g
Potassium chloride (KCl)	0.150g
Ammonium nitrate (NH_4NO_3)	1.000g
D-glucose	3.000g
p-Dimethylaminobenzenediazo sodium sulphonate	0.300g
Pentachloronitrobenzene	0.200g
Rose Bengal (tetrachlorotetradiodofluorescein)	0.150g
Purified agar	20.00g
added after autoclaving:	
Chloramphenicol	0.250g

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Appendix B: Chemicals, Reagents and Suppliers

This section provides supplier and product information for chemical compounds and reagents referred to in sections 2.2, 3.2, 4.2 and 5.2 (methods sections).

Compound	Supplier
Agar (purified) (L28)	Oxoid Ltd., Basingstoke, UK
Agarose	Sigma-Aldrich Co. Ltd, Dorset, UK.
Ammonium nitrate	Fisher Scientific UK Ltd., Leicester, UK.
L-Asparagine	Sigma-Aldrich, UK.
Benomyl	DuPont (UK) Ltd., Stevenage, UK.
Bromocresol green	Sigma-Aldrich, UK.
Bromophenol blue	Sigma-Aldrich, UK.
Boric acid (borate)	-
Calcium nitrate	BDH Lab. Supplies, Dorset, UK.
Chloramphenicol	Sigma-Aldrich, UK.
Chloroform:isoamyl alcohol (24:1)	Sigma-Aldrich, UK.
Copper sulphate	Sigma-Aldrich, UK.
Dipotassium hydrogen orthophosphate	BDH Lab. Supplies, UK.
dNTP's	Quantum Appligene, UK
EDTA (diaminoethanetetra-acetic acid disodium salt)	Sigma-Aldrich, UK.
Ethidium bromide	Sigma-Aldrich, UK.
Ethyl alcohol (ethanol)	Sigma-Aldrich, UK.
Gallic acid (1,2,4,5-Trihydroxybenzoic acid)	Sigma-Aldrich, UK.
Glacial acetic acid	-
Glucose (D-glucose)	-
Glycerol	Sigma-Aldrich, UK.
Hydrochloric acid	-
Hydrogen peroxide (100 volume)	-
Iron sulphate	BDH Lab. Supplies, UK.
Isopropyl alcohol (propan-2-ol)	Sigma-Aldrich, UK.
Loading dye (blue/orange)	Promega UK. Ltd., Southampton, UK.
Magnesium sulphate	BDH Lab. Supplies, UK.
Malt extract (L39)	Oxoid Ltd., UK
Malt extract agar (CM59)	Oxoid Ltd., UK
Manganese acetate	BDH Lab. Supplies, UK.
Mineral oil	Sigma-Aldrich, UK.
Methyl red	Sigma-Aldrich, UK.
Molecular markers (500 bp ladder)	Quantum Appligene, UK
Molecular markers (pBR322 Taq I/Hae III mix)	Quantum Appligene, UK
Nutrient agar (CM3)	Oxoid Ltd., UK

Nutrient broth (CM01)	Oxoid Ltd., UK
PAS (p-Aminosalicyclic acid)	Sigma-Aldrich, UK.
PCR Master Mix	Promega, UK.
p-Dimethylaminobenzenediazo sodium sulphonate	Sigma-Aldrich, UK.
Pentachloronitrobenzene	Sigma-Aldrich, UK.
Phenol:chloroform:isoamyl alcohol (25:24:1)	Sigma-Aldrich, UK.
Potassium chloride	BDH Lab. Supplies, UK.
Potassium dihydrogen orthophosphate	BDH Lab. Supplies, UK.
Puritabs Maxi (sodium dichloro-s-triazinetrione 425mg)	Schering-Plough Ltd. Mildenhall, Suffolk, U.K.
RAPD primer kit D (Operon Technologies)	VHBio Ltd., Chester-le-Street, Co. Durham, UK.
Rose Bengal (tetrachlorotetradiodofluorescein)	Sigma-Aldrich, UK.
Seesand (Fluka*)	Sigma-Aldrich, UK.
SDS (sodium dodecyl sulphate)	Sigma-Aldrich, UK.
Sodium acetate	Sigma-Aldrich, UK.
Sodium chloride	Fisher Scientific UK, UK.
Streptomycin sulphate	Sigma-Aldrich, UK.
Sulphuric acid	-
Tanalith CCA (3%)	Hicksons UK., Castleford, UK.
Taq DNA polymerase (+ [10 x] Reaction buffer)	Promega, UK.
TNS (Tris-*iso*-propyl-naphthalene-sulfonic acid sodium salt))	Acros Organics, New Jersey, US.
Tris-HCl (Tris[hydroxymethyl]-amino methane hydrochloride)	Sigma-Aldrich, UK.
Water (HPLC grade)	Sigma-Aldrich, UK.
Water (nuclease-free)	Promega, UK.
Zinc nitrate	BDH Lab. Supplies, UK.

*Fluka/Reidel-de Haen are subsidiaries of Sigma-Aldrich Co.

Appendix C: Determination of soil water-holding capacity (WHC)

Water-holding capacity of soil was determined by the method described in European standard ENV 807 "Wood preservatives - Determination of the toxic effectiveness against soft rotting micro-fungi and other soil inhabiting micro-organisms" (CEN, 2001). This measures the ability of a sample of soil or other test substrates to retain moisture.

Three soil samples of 200g were weighed and the weight of each was recorded (m_1). A small quantity of water was added to each sample until the soil particles began to form a crumb structure. A further 25ml of water was added to each sample, these were then mixed and left to stand for 2 hours.

A coarse filter paper was placed in the base of a Buchner funnel and a small amount of water added to produce a seal. The soil sample was poured into the funnel and spread evenly across the filter paper. Suction was applied slowly (to avoid perforating the filter paper) and when no more water could be removed from the sample, the suction was stopped. The sample and the filter paper were transferred to a container of known mass (m_2) and weighed (m_5). The container and sample were placed in an oven at 103°C ($\pm$ 2), dried overnight and re-weighed (m_4). The weight of a wet filter paper after the application of suction was determined (m_6), and the filter paper was oven-dried and re-weighed (m_3). The procedure was then repeated with the other two soil samples.

The following calculations were used to determine the water holding capacity of the soil:

Initial moisture content of sample (W_1)

$$= \frac{(m_1 + m_2 + m_3) - m_4}{m_4 - (m_2 + m_3)} \times 100$$

Moisture content of sample at water holding capacity (W_2)

$$= \frac{(m_3 + m_5) - (m_4 + m_6)}{m_4 - (m_2 + m_3)} \times 100$$

Amount of water required to raise moisture content of substrate to a given percentage water holding capacity

$$= \frac{[(W_3 - W_2)/100] - W_1}{100 + W_1} \times 100$$

(W_3 = percentage WHC required)

Calibration of soil moisture meter

Using the method described above, soil samples were set up at 20%, 40%, 60%, 80% and 100% WHC (as described in Hainey, 1992). These soils were used to calibrate the garden moisture probe used to monitor soil moisture content during the study.

References

CEN (2001)

European Standard ENV 807: Wood preservatives - Determination of the toxic effectiveness against soft rotting micro-fungi and other soil inhabiting micro-organisms. European Committee for Standardisation.

Hainey, S. D. (1992)

An investigation of the durability of U.K. grown softwood distribution poles CCA-treated by sap-displacement.

Ph.D. thesis (CNAA), Dundee Institute of Technology, Dundee, U.K., 258pp.

Appendix D: Statistical Analyses of Selected Field Trial Results

Results of ANOVA statistical evaluation of selected field and fungal cellar test results; results analysed for statistical significance at $p \geq 0.05$ using Dunnets' test. Three sets of comparisons were undertaken on each of the four selected measurements:

(i) Effect over time

Uplift 1 vs. Uplift 2; values that are statistically significantly different are indicated by ●, compared with the corresponding control denoted by ❍

(ii) Effect of environmental conditions

Field trial vs. Fungal cellar; ■ indicates values that are statistically significantly different from the corresponding control group indicated by the second symbol □.

(iii) Effect of treatment

T60 vs. CCA; CCA vs. untreated controls; T60 vs. untreated controls; using a similar system to those for (i) and (ii), statistically significant differences between T60 and CCA are indicated by the symbol pair ◆❖; CCA vs. untreated control by the symbol pair (▲△); T60 vs. untreated control by the symbol pair (●❍).

The measurements selected for statistical analysis were:

Subjective assessment scores – Table 4.3

Moisture content (recorded using moisture meter) – Table 4.4

Resistance to impact (pilodyn) – Table 4.5

Moisture content (oven-dry weights) – Table 4.8

Appendix D, part (i): Effect over time (Uplift 1 vs. Uplift 2)

Uplift 1		
PTF	**PPF**	**PF**
0.0 (0.0) ●	0.0 (0.0)	0.0 (0.0) ●

Uplift 2		
PTF	**PPF**	**PF**
1.4 (0.4) ○	0.0 (0.0)	2.4 (0.5) ○

Uplift 1		
PTC	**PPC**	**PC**
1.0 (0.5) ●	0.0 (0.0)	1.4 (0.4) ●

Uplift 2		
PTC	**PPC**	**PC**
2.5 (0.5) ○	0.0 (0.0)	2.8 (0.8) ○

Uplift 1		
STF	**SPF**	**SF**
0.0 (0.0) ●	0.0 (0.0)	0.2 (0.4) ●

Uplift 2		
STF	**SPF**	**SF**
1.2 (0.3) ○	0.0 (0.0)	1.8 (0.3) ○

Uplift 1		
STC	**SPC**	**SC**
0.8 (0.4) ●	0.0 (0.0)	1.2 (0.6) ●

Uplift 2		
STC	**SPC**	**SC**
3.0 (0.0) ○	0.4 (0.5)	3.2 (0.8) ○

Table 4.3: Mean scores from subjective assessment of field and fungal cellar stakes immediately following uplift. A total of 5 stakes were assessed per treatment group. Figures in parentheses represent standard deviations. Statistical significance is indicated by the symbol pair ● ○

Uplift 1				Uplift 2		
PTF	PPF	PF		PTF	PPF	PF
12.8 (1.3) ●	13.7 (0.4) ●	11.3 (0.4) ●	top	24.5 (5.0) ○	24.4 (0.7) ○	27.8 (5.3) ○
28.8 (1.4) ●	29.1 (1.1) ●	29.3 (1.2) ●	middle	38.0 (2.3) ○	32.8 (1.7) ○	38.7 (5.9) ○
29.0 (0.2) ●	27.9 (1.4)	28.8 (1.7) ●	bottom	34.12 (1.1) ○	31.1 (2.2)	36.9 (2.5) ○

Uplift 1				Uplift 2		
PTC	PPC	PC		PTC	PPC	PC
26.0 (4.2)	24.4 (2.8)	18.6 (1.1)	top	36.7 (10.5)	23.7 (1.2)	21.6 (3.7)
56.2 (3.6)	35.1 (3.6)	57.5 (2.1) ●	middle	58.9 (5.0)	37.2 (1.6)	66.5 (4.7) ○
50.0 (1.9)	40.3 (5.7)	39.2 (5.4) ●	bottom	55.0 (5.5)	38.2 (2.8)	53.2 (6.5) ○

Uplift 1				Uplift 2		
STF	SPF	SF		STF	SPF	SF
13.2 (0.9) ●	16.0 (0.3) ●	13.8 (0.1) ●	top	19.7 (2.1) ○	31.8 (2.0) ○	23.5 (4.0) ○
29.9 (2.0) ●	35.6 (2.8)	29.5 (2.4) ●	middle	38.3 (2.0) ○	40.1 (4.2)	38.6 (4.8) ○
32.8 (2.0) ●	37.8 (1.7)	30.0 (2.4)	bottom	38.2 (1.4) ○	38.2 (1.6)	32.8 (0.9)

Uplift 1				Uplift 2		
STC	SPC	SC		STC	SPC	SC
41.8 (19.5)	33.4 (2.3) ●	24.0 (2.5)	top	34.8 (19.5)	29.8 (1.7) ○	21.5 (1.2)
65.4 (8.1)	43.4 (4.0)	54.3 (7.1) ●	middle	67.9 (5.1)	47.2 (4.9)	73.2 (6.8) ○
58.1 (4.8)	50.6 (4.6) ●	43.3 (2.1) ●	bottom	63.8 (2.2)	60.4 (5.5) ○	67.0 (2.4) ○

Table 4.4: Mean moisture contents of field and fungal cellar stakes measured immediately following uplift, using a moisture meter. Presented means are calculated from 5 readings per treatment group. Figures in parentheses represent standard deviations. Statistical significance is indicated by the symbol pair ● ○

Uplift 1				Uplift 2		
PTF	PPF	PF		PTF	PPF	PF
6.5 (0.8) ●	5.6 (0.6) ●	6.1 (0.9) ●	top	8.5 (1.4) ○	6.7 (1.4) ○	8.2 (0.8) ○
10.0 (1.2)	7.8 (1.3)	9.6 (1.0)	middle	10.0 (2.3)	8.8 (1.1)	11.1 (3.4)
10.7 (1.4) ●	9.1 (1.0)	9.7 (1.1)	bottom	9.4 (1.0) ○	8.8 (1.1)	10.0 (1.3)

Uplift 1				Uplift 2		
PTC	PPC	PC		PTC	PPC	PC
6.8 (1.3)	4.8 (0.8)	6.5 (0.6) ●	top	8.7 (3.1)	5.0 (0.6)	5.3 (1.2) ○
11.1 (2.1)	6.6 (1.2)	13.0 (4.7)	middle	12.2 (2.8)	6.9 (0.7)	16.4 (6.8)
11.6 (1.8)	7.4 (1.4)	12.0 (2.4)	bottom	11.9 (2.6)	7.5 (1.1)	13.9 (2.8)

Uplift 1				Uplift 2		
STF	SPF	SF		STF	SPF	SF
9.4 (1.5) ●	9.4 (2.1)	8.1 (1.4) ●	top	12.2 (1.2) ○	10.1 (2.9)	11.4 (2.3) ○
15.3 (2.6)	13.4 (1.6)	13.7 (3.5)	middle	16.7 (1.5)	13.4 (1.7)	16.1 (5.1)
16.2 (3.2) ●	13.9 (2.0)	13.4 (3.0)	bottom	11.8 (2.0) ○	12.4 (2.6)	14.8 (2.5)

Uplift 1				Uplift 2		
STC	SPC	SC		STC	SPC	SC
11.4 (2.1)	8.5 (1.4)	10.2 (2.0)	top	10.9 (2.2)	8.6 (1.6)	10.8 (1.2)
14.9 (1.9) ●	12.7 (1.9)	15.2 (5.8) ●	middle	17.9 (3.6) ○	11.2 (2.7)	24.2 (4.1) ○
15.2 (2.7)	13.1 (1.7)	15.7 (5.1)	bottom	14.4 (1.0)	12.8 (2.5)	19.1 (4.5)

Table 4.5: Resistance to impact measured using a pilodyn, with the depth of pin penetration measured in millimetres (mm). Values represent the mean of 10 readings. Figures in parentheses represent standard deviations. Statistical significance is indicated by the symbol pair ● ○

Uplift 1				Uplift 2		
PTF	**PPF**	**PF**		**PTF**	**PPF**	**PF**
18.1 (0.8) ●	15.0 (0.6) ●	16.4 (1.0) ●	**top**	46.2 (23.7) ○	21.7 (0.7) ○	46.3 (14.5) ○
56.2 (24.1) ●	23.2 (3.6) ●	41.3 (10.6) ●	**middle**	91.3 (28.7) ○	58.9 (24.0) ○	91.1 (25.6) ○
66.5 (20.0) ●	40.0 (21.8) ●	56.1 (15.2) ●	**bottom**	89.1 (21.0) ○	87.7 (38.1) ○	80.4 (20.3) ○

Uplift 1				Uplift 2		
PTC	**PPC**	**PC**		**PTC**	**PPC**	**PC**
45.3 (27.5) ●	23.1 (0.9) ●	22.6 (1.3) ●	**top**	71.4 (31.9) ○	20.3 (0.9) ○	18.7 (0.7) ○
114.3 (0.1)	28.6 (0.9)	35.9 (4.4) ●	**middle**	106. 8 (36.5)	28.9 (0.9)	53.0 (22.0) ○
108.8 (14.4)	39.6 (9.8)	34.3 (5.9) ●	**bottom**	108.6(28.4)	35.4 (7.3)	78.4 (34.2) ○

Uplift 1				Uplift 2		
STF	**SPF**	**SF**		**STF**	**SPF**	**SF**
13.9 (0.7) ●	16.7 (0.6) ●	14.2 (0.7) ●	**top**	23.0 (0.5) ○	22.2 (1.3) ○	26.7 (4.4) ○
24.9 (12.3) ●	53.8 (19.1) ●	36.9 (15.4) ●	**middle**	99.4 (49.1) ○	95.6 (31.1) ○	69.0 (35.9) ○
115.0 (50.5) ●	111.8 (39.0) ●	40.0 (33.5)	**bottom**	192.5 (32.8) ○	155.2 (31.0) ○	48.2 (20.6)

Uplift 1				Uplift 2		
STC	**SPC**	**SC**		**STC**	**SPC**	**SC**
43.0 (22.2)	27.0 (0.8) ●	24.6 (0.8) ●	**top**	33.2 (21.1)	17.4 (1.3) ○	16.9 (1.4) ○
92.8 (52.0) ●	41.0 (6.3) ●	35.0 (4.3) ●	**middle**	149.7 (75.8) ○	32.9 (8.8) ○	46.1 (25.8) ○
157.3 (61.6) ●	80.4 (34.2) ●	45.6 (16.2) ●	**bottom**	262.8 (42.6) ○	128.1 (27.1) ○	183.2 (73.7) ○

Table 4.8: Mean moisture contents (%) for each treatment group measured by dry weight analysis. Figures in parentheses represent standard deviations and each value represents a mean of 28 readings (7 sample locations on four replicate stakes). Statistical significance is indicated by the symbol pair ● ○

Appendix D, part (ii): Effect of environmental conditions (Field vs. Fungal cellar)

Uplift 1		
PTF	**PPF**	**PF**
0.0 (0.0) ■	0.0 (0.0)	0.0 (0.0) ■

Uplift 2		
PTF	**PPF**	**PF**
1.4 (0.4) ■	0.0 (0.0)	2.4 (0.5)

Uplift 1		
PTC	**PPC**	**PC**
1.0 (0.5) □	0.0 (0.0)	1.4 (0.4) □

Uplift 2		
PTC	**PPC**	**PC**
2.5 (0.5) □	0.0 (0.0)	2.8 (0.8)

Uplift 1		
STF	**SPF**	**SF**
0.0 (0.0) ■	0.0 (0.0)	0.2 (0.4) ■

Uplift 2		
STF	**SPF**	**SF**
1.2 (0.3) ■	0.0 (0.0)	1.8 (0.3) ■

Uplift 1		
STC	**SPC**	**SC**
0.8 (0.4) □	0.0 (0.0)	1.2 (0.6) □

Uplift 2		
STC	**SPC**	**SC**
3.0 (0.0) □	0.4 (0.5)	3.2 (0.8) □

Table 4.3: Mean scores from subjective assessment of field and fungal cellar stakes immediately following uplift. A total of 5 stakes were assessed per treatment group. Figures in parentheses represent standard deviations. Statistical significance is indicated by the symbol pair ■ □

Uplift 1				Uplift 2		
PTF	PPF	PF		PTF	PPF	PF
12.8 (1.3) ■	13.7 (0.4) ■	11.3 (0.4) ■	top	24.5 (5.0)	24.4 (0.7)	27.8 (5.3)
28.8 (1.4) ■	29.1 (1.1) ■	29.3 (1.2) ■	middle	38.0 (2.3) ■	32.8 (1.7) ■	38.7 (5.9) ■
29.0 (0.2) ■	27.9 (1.4) ■	28.8 (1.7) ■	bottom	34.12 (1.1) ■	31.1 (2.2) ■	36.9 (2.5) ■

Uplift 1				Uplift 2		
PTC	PPC	PC		PTC	PPC	PC
26.0 (4.2) □	24.4 (2.8) □	18.6 (1.1) □	top	36.7 (10.5)	23.7 (1.2)	21.6 (3.7)
56.2 (3.6) □	35.1 (3.6) □	57.5 (2.1) □	middle	58.9 (5.0) □	37.2 (1.6) □	66.5 (4.7) □
50.0 (1.9) □	40.3 (5.7) □	39.2 (5.4) □	bottom	55.0 (5.5) □	38.2 (2.8) □	53.2 (6.5) □

Uplift 1				Uplift 2		
STF	SPF	SF		STF	SPF	SF
13.2 (0.9) ■	16.0 (0.3) ■	13.8 (0.1) ■	top	19.7 (2.1)	31.8 (2.0)	23.5 (4.0)
29.9 (2.0) ■	35.6 (2.8) ■	29.5 (2.4) ■	middle	38.3 (2.0) ■	40.1 (4.2)	38.6 (4.8) ■
32.8 (2.0) ■	37.8 (1.7) ■	30.0 (2.4) ■	bottom	38.2 (1.4) ■	38.2 (1.6) ■	32.8 (0.9) ■

Uplift 1				Uplift 2		
STC	SPC	SC		STC	SPC	SC
41.8 (19.5) □	33.4 (2.3) □	24.0 (2.5) □	top	34.8 (19.5)	29.8 (1.7)	21.5 (1.2)
65.4 (8.1) □	43.4 (4.0) □	54.3 (7.1) □	middle	67.9 (5.1) □	47.2 (4.9)	73.2 (6.8) □
58.1 (4.8) □	50.6 (4.6) □	43.3 (2.1) □	bottom	63.8 (2.2) □	60.4 (5.5) □	67.0 (2.4) □

Table 4.4: Mean moisture contents of field and fungal cellar stakes measured immediately following uplift, using a moisture meter. Presented means are calculated from 5 readings per treatment group. Figures in parentheses represent standard deviations. Statistical significance is indicated by the symbol pair ■ □

Uplift 1				Uplift 2		
PTF	PPF	PF		PTF	PPF	PF
6.5 (0.8)	5.6 (0.6) ■	6.1 (0.9)	top	8.5 (1.4)	6.7 (1.4) ■	8.2 (0.8) ■
10.0 (1.2)	7.8 (1.3)	9.6 (1.0) ■	middle	10.0 (2.3)	8.8 (1.1) ■	11.1 (3.4) ■
10.7 (1.4)	9.1 (1.0) ■	9.7 (1.1) ■	bottom	9.4 (1.0) ■	8.8 (1.1) ■	10.0 (1.3) ■

Uplift 1				Uplift 2		
PTC	PPC	PC		PTC	PPC	PC
6.8 (1.3)	4.8 (0.8) □	6.5 (0.6)	top	8.7 (3.1)	5.0 (0.6) □	5.3 (1.2) □
11.1 (2.1)	6.6 (1.2)	13.0 (4.7) □	middle	12.2 (2.8)	6.9 (0.7) □	16.4 (6.8) □
11.6 (1.8)	7.4 (1.4) □	12.0 (2.4) □	bottom	11.9 (2.6) □	7.5 (1.1) □	13.9 (2.8) □

Uplift 1				Uplift 2		
STF	SPF	SF		STF	SPF	SF
9.4 (1.5) ■	9.4 (2.1)	8.1 (1.4) ■	top	12.2 (1.2)	10. 1 (2.9)	11.4 (2.3)
15.3 (2.6)	13.4 (1.6)	13.7 (3.5)	middle	16.7 (1.5)	13.4 (1.7)	16.1 (5.1) ■
16.2 (3.2)	13.9 (2.0)	13.4 (3.0)	bottom	11.8 (2.0) ■	12.4 (2.6)	14.8 (2.5) ■

Uplift 1				Uplift 2		
STC	SPC	SC		STC	SPC	SC
11.4 (2.1) □	8.5 (1.4)	10.2 (2.0) □	top	10.9 (2.2)	8.6 (1.6)	10.8 (1.2)
14.9 (1.9)	12.7 (1.9)	15.2 (5.8)	middle	17.9 (3.6)	11.2 (2.7)	24.2 (4.1) □
15.2 (2.7)	13.1 (1.7)	15.7 (5.1)	bottom	14.4 (1.0) □	12.8 (2.5)	19.1 (4.5) □

Table 4.5: Resistance to impact measured using a pilodyn, with the depth of pin penetration measured in millimetres (mm). Values represent the mean of 10 readings. Figures in parentheses represent standard deviations. Statistical significance is indicated by the symbol pair ■ □

Uplift 1				Uplift 2		
PTF	PPF	PF		PTF	PPF	PF
18.1 (0.8) ■	15.0 (0.6) ■	16.4 (1.0) ■	top	46.2 (23.7) ■	21.7 (0.7) ■	46.3 (14.5) ■
56.2 (24.1) ■	23.2 (3.6) ■	41.3 (10.6) ■	middle	91.3 (28.7) ■	58.9 (24.0) ■	91.1 (25.6) ■
66.5 (20.0) ■	40.0 (21.8)	56.1 (15.2) ■	bottom	89.1 (21.0) ■	87.7 (38.1) ■	80.4 (20.3)

Uplift 1				Uplift 2		
PTC	PPC	PC		PTC	PPC	PC
45.3 (27.5) □	23.1 (0.9) □	22.6 (1.3) □	top	71.4 (31.9) □	20.3 (0.9) □	18.7 (0.7) □
114.3 (0.1) □	28.6 (0.9) □	35.9 (4.4) □	middle	106. 8 (36.5) □	28.9 (0.9) □	53.0 (22.0) □
108.8 (14.4) □	39.6 (9.8)	34.3 (5.9) □	bottom	108.6(28.4) □	35.4 (7.3) □	78.4 (34.2)

Uplift 1				Uplift 2		
STF	SPF	SF		STF	SPF	SF
13.9 (0.7) ■	16.7 (0.6) ■	14.2 (0.7) ■	top	23.0 (0.5) ■	22.2 (1.3) ■	26.7 (4.4) ■
24.9 (12.3) ■	53.8 (19.1) ■	36.9 (15.4)	middle	99.4 (49.1) ■	95.6 (31.1) ■	69.0 (35.9) ■
115.0 (50.5) ■	111.8 (39.0) ■	40.0 (33.5)	bottom	192.5 (32.8) ■	155.2 (31.0) ■	48.2 (20.6) ■

Uplift 1				Uplift 2		
STC	SPC	SC		STC	SPC	SC
43.0 (22.2) □	27.0 (0.8) □	24.6 (0.8) □	top	33.2 (21.1) □	17.4 (1.3) □	16.9 (1.4) □
92.8 (52.0) □	41.0 (6.3) □	35.0 (4.3)	middle	149.7 (75.8) □	32.9 (8.8) □	46.1 (25.8) □
157.3 (61.6) □	80.4 (34.2) □	45.6 (16.2)	bottom	262.8 (42.6) □	128.1 (27.1) □	183.2 (73.7) □

Table 4.8: Mean moisture contents (%) for each treatment group measured by dry weight analysis. Figures in parentheses represent standard deviations and each value represents a mean of 28 readings (7 sample locations on four replicate stakes). Statistical significance is indicated by the symbol pair ■ □

Appendix D, part (iii): Effect of treatment (T60; CCA; untreated controls)

Uplift 1		
PTF	**PPF**	**PF**
0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

Uplift 2		
PTF	**PPF**	**PF**
1.4 (0.4) ◆●	0.0 (0.0) ❖▲	2.4 (0.5) △❍

Uplift 1		
PTC	**PPC**	**PC**
1.0 (0.5) ◆	0.0 (0.0) ❖▲	1.4 (0.4) △

Uplift 2		
PTC	**PPC**	**PC**
2.5 (0.5) ◆	0.0 (0.0) ❖▲	2.8 (0.8) △

Uplift 1		
STF	**SPF**	**SF**
0.0 (0.0) ◆	0.0 (0.0) ❖	0.2 (0.4)

Uplift 2		
STF	**SPF**	**SF**
1.2 (0.3) ◆	0.0 (0.0) ❖▲	1.8 (0.3) △

Uplift 1		
STC	**SPC**	**SC**
0.8 (0.4) ◆	0.0 (0.0) ❖▲	1.2 (0.6) △

Uplift 2		
STC	**SPC**	**SC**
3.0 (0.0) ◆	0.4 (0.5) ❖▲	3.2 (0.8) △

Table 4.3: Mean scores from subjective assessment of field and fungal cellar stakes immediately following uplift. A total of 5 stakes were assessed per treatment group. Figures in parentheses represent standard deviations.
T60 vs. CCA (◆❖); T60 vs. untreated control (●❍); CCA vs. untreated control (▲△)

Uplift 1				Uplift 2		
PTF	PPF	PF		PTF	PPF	PF
12.8 (1.3)	13.7 (0.4) ▲	11.3 (0.4) △	top	24.5 (5.0)	24.4 (0.7)	27.8 (5.3)
28.8 (1.4)	29.1 (1.1)	29.3 (1.2)	middle	38.0 (2.3) ◆	32.8 (1.7) ❖	38.7 (5.9)
29.0 (0.2)	27.9 (1.4)	28.8 (1.7)	bottom	34.12 (1.1) ◆	31.1 (2.2) ❖▲	36.9 (2.5) △

Uplift 1				Uplift 2		
PTC	PPC	PC		PTC	PPC	PC
26.0 (4.2) ●	24.4 (2.8) ▲	18.6 (1.1) ○ △	top	36.7 (10.5) ◆●	23.7 (1.2) ❖	21.6 (3.7) ○
56.2 (3.6) ◆	35.1 (3.6) ❖▲	57.5 (2.1) △	middle	58.9 (5.0) ◆	37.2 (1.6) ❖▲	66.5 (4.7) △
50.0 (1.9) ◆●	40.3 (5.7) ❖	39.2 (5.4) ○	bottom	55.0 (5.5) ◆	38.2 (2.8) ❖▲	53.2 (6.5) △

Uplift 1				Uplift 2		
STF	SPF	SF		STF	SPF	SF
13.2 (0.9) ◆	16.0 (0.3) ❖ ▲	13.8 (0.1) △	top	19.7 (2.1) ◆	31.8 (2.0) ❖▲	23.5 (4.0) △
29.9 (2.0) ◆	35.6 (2.8) ❖ ▲	29.5 (2.4) △	middle	38.3 (2.0)	40.1 (4.2)	38.6 (4.8)
32.8 (2.0) ◆	37.8 (1.7) ❖ ▲	30.0 (2.4) △	bottom	38.2 (1.4) ●	38.2 (1.6) ▲	32.8 (0.9) ○△

Uplift 1				Uplift 2		
STC	SPC	SC		STC	SPC	SC
41.8 (19.5)	33.4 (2.3) ▲	24.0 (2.5) △	top	34.8 (19.5)	29.8 (1.7) ▲	21.5 (1.2) △
65.4 (8.1) ◆	43.4 (4.0) ❖▲	54.3 (7.1) △	middle	67.9 (5.1) ◆	47.2 (4.9) ❖▲	73.2 (6.8) △
58.1 (4.8) ●	50.6 (4.6) ▲	43.3 (2.1) ○△	bottom	63.8 (2.2)	60.4 (5.5)	67.0 (2.4)

Table 4.4: Mean moisture contents of field and fungal cellar stakes measured immediately following uplift, using a moisture meter. Presented means are calculated from 5 readings per treatment group. Figures in parentheses represent standard deviations. See Table 4.3 for key.

Uplift 1				Uplift 2		
PTF	PPF	PF		PTF	PPF	PF
6.5 (0.8) ◆	5.6 (0.6) ❖	6.1 (0.9)	top	8.5 (1.4) ◆	6.7 (1.4) ❖▲	8.2 (0.8) △
10.0 (1.2) ◆	7.8 (1.3) ❖▲	9.6 (1.0) △	middle	10.0 (2.3)	8.8 (1.1)	11.1 (3.4)
10.7 (1.4) ◆	9.1 (1.0) ❖	9.7 (1.1)	bottom	9.4 (1.0)	8.8 (1.1)	10.0 (1.3)

Uplift 1				Uplift 2		
PTC	PPC	PC		PTC	PPC	PC
6.8 (1.3) ◆	4.8 (0.8) ❖▲	6.5 (0.6) △	top	8.7 (3.1) ◆●	5.0 (0.6) ❖	5.3 (1.2) ○
11.1 (2.1) ◆	6.6 (1.2) ❖▲	13.0 (4.7) △	middle	12.2 (2.8) ◆	6.9 (0.7) ❖▲	16.4 (6.8) △
11.6 (1.8) ◆	7.4 (1.4) ❖▲	12.0 (2.4) △	bottom	11.9 (2.6) ◆	7.5 (1.1) ❖▲	13.9 (2.8) △

Uplift 1				Uplift 2		
STF	SPF	SF		STF	SPF	SF
9.4 (1.5)	9.4 (2.1)	8.1 (1.4)	top	12.2 (1.2)	10.1 (2.9)	11.4 (2.3)
15.3 (2.6)	13.4 (1.6)	13.7 (3.5)	middle	16.7 (1.5) ◆	13.4 (1.7) ❖	16.1 (5.1)
16.2 (3.2)	13.9 (2.0)	13.4 (3.0)	bottom	11.8 (2.0) ●	12.4 (2.6)	14.8 (2.5) ○

Uplift 1				Uplift 2		
STC	SPC	SC		STC	SPC	SC
11.4 (2.1) ◆	8.5 (1.4) ❖▲	10.2 (2.0) △	top	10.9 (2.2) ◆	8.6 (1.6) ❖▲	10.8 (1.2) △
14.9 (1.9) ◆	12.7 (1.9) ❖	15.2 (5.8)	middle	17.9 (3.6) ◆●	11.2 (2.7) ❖▲	24.2 (4.1) ○△
15.2 (2.7)	13.1 (1.7)	15.7 (5.1)	bottom	14.4 (1.0) ●	12.8 (2.5) ▲	19.1 (4.5) ○△

Table 4.5: Resistance to impact measured using a pilodyn, with the depth of pin penetration measured in millimetres (mm). Values represent the mean of 10 readings. Figures in parentheses represent standard deviations.
T60 vs. CCA (◆❖); T60 vs. untreated control (●○); CCA vs. untreated control (▲△)

Uplift 1				Uplift 2		
PTF	PPF	PF		PTF	PPF	PF
18.1 (0.8) ●◆	15.0 (0.6) ▲❖	16.4 (1.0) ○△	top	46.2 (23.7) ◆	21.7 (0.7) ▲❖	46.3 (14.5) △
56.2 (24.1) ●◆	23.2 (3.6) ▲❖	41.3 (10.6) ○△	middle	91.3 (28.7) ◆	58.9 (24.0) ▲❖	91.1 (25.6) △
66.5 (20.0) ●◆	40.0 (21.8) ▲❖	56.1 (15.2) ○△	bottom	89.1 (21.0)	87.7 (38.1)	80.4 (20.3)

Uplift 1				Uplift 2		
PTC	PPC	PC		PTC	PPC	PC
45.3 (27.5) ●◆	23.1 (0.9) ❖	22.6 (1.3) ○	top	71.4 (31.9) ●◆	20.3 (0.9) ▲❖	18.7 (0.7) ○△
114.3 (0.1) ●◆	28.6 (0.9) ▲❖	35.9 (4.4) ○△	middle	106. 8 (36.5) ●◆	28.9 (0.9) ▲❖	53.0 (22.0) ○△
108.8 (14.4) ●◆	39.6 (9.8) ▲❖	34.3 (5.9) ○△	bottom	108.6(28.4) ●◆	35.4 (7.3) ▲❖	78.4 (34.2) ○△

Uplift 1				Uplift 2		
STF	SPF	SF		STF	SPF	SF
13.9 (0.7) ◆	16.7 (0.6) ▲❖	14.2 (0.7) △	top	23.0 (0.5) ●◆	22.2 (1.3) ▲❖	26.7 (4.4) ○△
24.9 (12.3) ●◆	53.8 (19.1) ▲❖	36.9 (15.4) ○△	middle	99.4 (49.1) ●	95.6 (31.1) ▲	69.0 (35.9) ○△
115.0 (50.5) ●	111.8 (39.0) ▲	40.0 (33.5) ○△	bottom	192.5 (32.8) ●◆	155.2 (31.0) ▲❖	48.2 (20.6) ○△

Uplift 1				Uplift 2		
STC	SPC	SC		STC	SPC	SC
43.0 (22.2) ●◆	27.0 (0.8) ▲❖	24.6 (0.8) ○△	top	33.2 (21.1) ●◆	17.4 (1.3) ❖	16.9 (1.4) ○
92.8 (52.0) ●◆	41.0 (6.3) ▲❖	35.0 (4.3) ○△	middle	149.7 (75.8) ●◆	32.9 (8.8) ▲❖	46.1 (25.8) ○△
157.3 (61.6) ●◆	80.4 (34.2) ▲❖	45.6 (16.2) ○△	bottom	262.8 (42.6) ●◆	128.1 (27.1) ▲❖	183.2 (73.7) ○△

Table 4.8: Mean moisture contents (%) for each treatment group measured by dry weight analysis. Figures in parentheses represent standard deviations and each value represents a mean of 28 readings (7 sample locations on four replicate stakes).
T60 vs. CCA (◆❖); T60 vs. untreated control (●○); CCA vs. untreated control (▲△)

Development of molecular detection methods for research in biocontrol of wood decay.

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THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

SECTION 1 BIOLOGY

Development of Molecular Detection Methods for Research in Biocontrol of Wood Decay

by

Heather L. Brown and Alan Bruce

Scottish Institute for Wood Technology
Department of Molecular and Life Sciences
University of Abertay Dundee
Bell Street, Dundee
DD1 1HG
Scotland, U.K.

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IRG Secretariat
KTH
Brinellvägen 34
S-100 44 Stockholm
Sweden

DEVELOPMENT OF MOLECULAR DETECTION METHODS FOR RESEARCH IN BIOCONTROL OF WOOD DECAY

Heather L. Brown and Alan Bruce
Scottish Institute for Wood Technology
University of Abertay Dundee, Scotland UK

ABSTRACT

Field and fungal cellar trials have been set up to assess the biocontrol potential of a selected *Trichoderma viride* isolate in a situation representative of the end use of treated timber in ground contact situations. These trials are designed to give information about the efficacy of biological control as well as the suitability of existing chemical treatment methods for use with biocontrol fungi.

An essential requirement in such trials is to be able to assess the extent of fungal pretreatment of wooden stakes and also to continually monitor the biological control isolate during the exposure period in soil. Suitable molecular-based PCR (polymerase chain reaction) systems may achieve both of these objectives through the development of appropriate primers for the *Trichoderma* isolate and by measurement of the intensity of DNA bands produced after PCR processing of field samples.

This paper presents results from PCR analysis of wood samples removed from Sitka spruce treated with *Trichoderma* spores in a pressure impregnation plant. Various short DNA primers were tested and the most appropriate one selected for quantitative analysis of the *Trichoderma*-treated wood. The paper discusses the development of the techniques and the implications for the use of such PCR methods as molecular detection systems in the field of wood biocontrol.

Key Word(s): Biological control; *Trichoderma*; Polymerase chain reaction (PCR); Fungal detection

INTRODUCTION

The traditional method of preparing timber for use in ground contact situations is to apply toxic chemicals such as creosote or copper-chrome-arsenic (CCA) formulations. The efficacy of creosote and CCA treatment is well documented, and the methods used for chemical impregnation are tried and tested (Greaves, 1987). However, some chemicals previously used as preservatives for selected ground contact timbers have been seen to fail during their expected service period (Schmidt and Kerner-Gang, 1986) and in addition the safety of some chemical preservatives is now under increasing scrutiny (Barnes, 1993).

One possible alternative to chemical treatment is the use of biological control systems. Although laboratory-based experiments are adequate for screening potential biocontrol agents, the most appropriate way to assess the full potential of a biocontrol agent is through field trials. Previously reported work (Tucker *et al*, 1996) has shown that certain isolates of *Trichoderma* have a protective effect against basidiomycete decay fungi when tested using wood block systems based on European and American standard test methods developed for chemical preservatives. A large-scale experiment designed to assess the biocontrol potential of a single *Trichoderma viride* isolate (T60) has been set up in a field site near Dundee in Scotland.

In order for biological control to become a viable alternative to chemical preservation, it is necessary, in addition to establishing successful field performance, to investigate other aspects such as efficacy of the delivery system and the influence of the organism on the microbial ecology of the wood-soil interface. Traditional chemical treatments employ vacuum-pressure impregnation as a means of ensuring the maximum possible penetration of chemical formulations into the timber. If biological control is to become an acceptable alternative to chemical preservation for certain wood products, then it would be attractive if the mode of delivery of biological agents was compatible with standard industrial processes for chemical application. The use of fungal spores as biocontrol agents has already been investigated in the remedial treatment of *Serpula lacrymans*-infected wood by spraying a spore suspension of *Trichoderma harzianum* (Score, pers. comm.); and in the biocontrol of basidiomycete decay fungi, by treating wood blocks with a *Trichoderma* spore suspension using a bench-top pressure impregnation system (Tucker *et al*, 1997). Studies at this institute have shown that it is possible to treat wood with *Trichoderma* spores using a pilot preservation plant and commercial treating conditions without adversely affecting subsequent germination of the fungal spores. However quantification of the levels of *Trichoderma* spores applied to the surface or indeed throughout the stakes has been difficult. This paper describes the development of a molecular method to quantify the levels of *Trichoderma* spores in treated wooden stakes. The concentration of spores impregnated into stakes and thereby the efficacy and uniformity of pressure impregnation to treat wood with fungal spores can therefore be assessed.

Since the use of biological control agents is not yet an established technology for protecting timber from fungal attack, potential isolates need to be closely monitored to

ensure that alterations to any natural pattern of microbial succession within the wooden stakes in the field can be carefully identified. It is important to closely monitor the biocontrol agent (in this case *Trichoderma viride* isolate T60) in both wood and adjacent soil to determine colonisation and spread of the fungus.

Several different methods can be used for the detection and identification of fungi, ranging from isolation and culturing using selective media to biochemical or molecular techniques such as enzyme assays and immunological-based systems such as enzyme linked immunosorbent assays (ELISA). However, many of these methods are limited in terms of their specificity, making them unsuitable for the application described here. One molecular detection method with the potential to detect and quantify specific strains of microorganisms is the polymerase chain reaction (PCR). There are many publications detailing the use of PCR to distinguish between different strains of the same fungal species. For example, Zimand *et al* (1994) used RAPD (randomly amplified polymorphic DNA) PCR to differentiate between different strains of *Trichoderma*; and Arisan-Atac *et al* (1995) identified subgroups of *T. viride* through RAPD PCR. In addition to PCR being used to discriminate between closely related strains of the same genus, Schlick *et al* (1994) have used DNA and PCR fingerprinting to distinguish mutant strains derived from the same isolate, even though the ITS (internal transcribed spacers) sequences are identical. Internal Transcribed Spacers are regions of the rDNA gene complex, the repeat units of which vary among species within a genus and they can therefore be used for species identification and differentiation.

This paper describes the early development of a PCR system for the specific detection and quantification of *Trichoderma* isolates for use as biological control agents. The objectives of the work described here were to assess the distribution of fungal spores in wood following pressure impregnation, and to develop and assess a method for detecting and quantifying spores of a specific fungal isolate.

METHOD

Sample Preparation

Wooden stakes of Scots pine (*Pinus sylvestris* L.) and Sitka spruce (*Picea sitchensis* (Borg) Carr) measuring 500 mm x 50 mm x 25 mm were pressure impregnated with a suspension of *T. viride* (isolate T60) spores using a pilot preservation plant (Hickson Timber Products Ltd., Castleford, W. Yorkshire, UK), at a concentration of 8×10^5 spores/ml. The treatment cycle used for both wood species was P3 *i.e.* that recommended by the manufacturer for Scots pine sawn wood to be used as ground contact timbers (BS 4072 (1987), BS 5589 (1989)). This consisted of an initial vacuum of 600 mmHg for 30 minutes followed by a 90 minute pressure period at 12.8 Kg/cm^3. No final vacuum was drawn to avoid the removal of any spores from the wood surface. Fifteen samples were cut from each of 5 stakes of Scots pine and 5 stakes of Sitka spruce as shown in **Fig. 1**.

These were milled in a Spex 6700 freezer mill (Glen Creston, Stanmore, Middlesex, UK). The milled samples were then frozen at -20°C and stored until required.

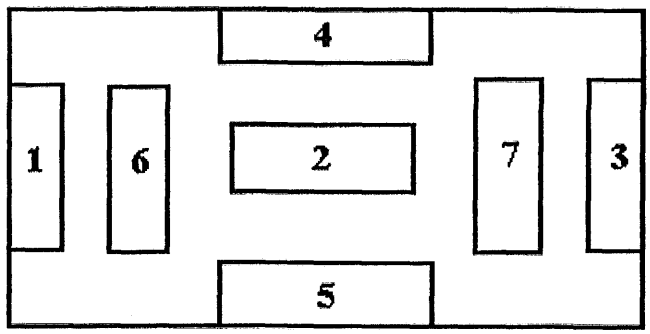


Fig. 1: Diagram showing sample numbers corresponding to cross-sectional areas on a wooden stake

Samples 1, 3, 4 and 5 are blocks taken from the outside of the stake, to a depth of 5 mm. Samples 6 and 7 are intermediate blocks, taken from a depth of 10-15 mm from the 2.5 cm face of the stake (but only 5 mm from the surface of the 5 cm face of the stake). Sample 2 is from the centre of the stake, a depth of 20 mm from the 5 cm face of the stake. The samples were taken from three areas along the length of the stake - top, middle and bottom.

DNA Extraction

The extraction procedure used was adapted from Lee and Taylor (1990). Approximately 0.5 ml by volume of each milled wood sample was placed in sterile Eppendorf microcentrifuge tubes, and 400 µl of lysis buffer added. Lysis buffer contained 50 mM Tris-HCl (pH 7.2), 50 mM EDTA (diaminoethanetetra-acetic acid), 3% SDS (sodium dodecyl sulphate), 1% β-mercaptoethanol in sterile distilled water. The tubes were vortexed to mix the contents and then dipped in liquid nitrogen to freeze the sample. The tubes were incubated in a water bath at 65°C for 1 hour, being dipped in liquid nitrogen every 20-30 minutes during incubation. The purpose of this step was to disrupt cell membranes. Four hundred microlitres of 25:24:1 phenol:chloroform:isoamyl alcohol was added and the tubes were vortexed. The samples were then microcentrifuged at 10,000 x g (13,000 rpm) for 15 minutes at room temperature until the aqueous phase was clear. The aqueous phase (containing any DNA) was removed to a fresh Eppendorf tube and an equal amount of 24:1 chloroform:isoamyl alcohol was added. The tubes were then vortexed and the aqueous phase again transferred to a fresh tube. Ten microlitres of 3M sodium acetate was added to the aqueous phase followed by the addition of 0.54 volumes (i.e. sample volume x 0.54) of isopropanol. The tubes' contents were mixed by inverting gently, followed by microcentrifuging at 10,000 x g (13,000 rpm) for 2 minutes. The supernatant was decanted and the remaining pellet (DNA) rinsed with 70% ethanol. The tubes were inverted for 1 minute and drained onto sterile paper towel. The DNA pellets

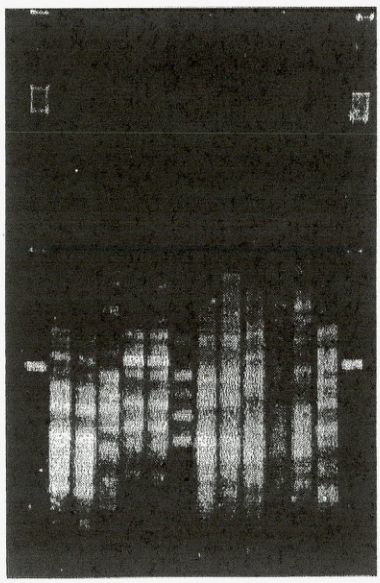
were left to dry at room temperature and resuspended in 100 µl of sterile distilled water, then stored at 4°C until used for PCR.

Determination of Optimal DNA Concentration

Using a primer already known to detect *Trichoderma* DNA (GGGACGTTGG: Score, pers. comm.), PCR tubes were set up containing differing amounts of DNA *i.e.* 5 µl, 7.5 µl, 10 µl and 12.5 µl in 100 µl reaction volumes. The amount of water added to each tube was reduced to compensate for the increased amount of DNA solution. This was done to evaluate the most appropriate amount of DNA to add to the reaction for optimal amplification.

Primer Selection

An important early stage in the development of a PCR system is to identify a primer which will provide a reproducible and easy to interpret banding pattern (or profile). A RAPD primer is a short oligonucleotide (usually around 10 base pairs in length) which anneals to a complementary site on the target DNA and provides a starting point for DNA polymerisation. The primers used were oligonucleotides from the Operon Technologies Inc. RAPD primer kit D (VH Bio Ltd., Chester-Le-Street, Co. Durham). Primers 1, 2, 3 and 8 were tested to select the one which gave the most appropriate profile for *Trichoderma viride* isolate T60 (see **Fig. 2**).



1 - - → 14

Lane 1 marker (500bp ladder)
Lane 2 OPD-01, T60
Lane 3 OPD-01, T60
Lane 4 OPD-01, control
Lane 5 OPD-02, T60
Lane 6 OPD-02, T60
Lane 7 OPD-02, control
Lane 8 OPD-03, T60
Lane 9 OPD-03, T60
Lane 10 OPD-03, control
Lane 11 OPD-08, T60
Lane 12 OPD-08, T60
Lane 13 OPD-08, control
Lane 14 marker (500bp ladder)

Fig. 2: Gel showing detection of T60 by primers OPD-01, -02, -03 and -08

Polymerase Chain Reaction

The PCR protocol followed was adapted from Theodore *et al* (1995). Reaction tubes were set up containing the following:-

100 µl volume reaction: 60 µl sterile distilled water, 16 µl nucleotide mixture (1.25 mM of each nucleotide), 8 µl primer (OPD-02), 10 µl *Taq* buffer with magnesium chloride (10 x concentration), 1 µl *Taq* polymerase solution (*Taq* polymerase is a heat-stable enzyme extracted from the thermophilic bacteria *Thermus aquaticus*. It is used in PCR as it does not denature at the high temperatures needed for the reaction), 30 µl mineral oil (this is used as a thermal barrier), 5 µl DNA solution (extracted from the treated wooden stakes).

25 µl volume reaction: 11 µl sterile distilled water, 8 µl nucleotide mixture (1.25 mM of each nucleotide), 2 µl primer (OPD-02), 2.5 µl *Taq* buffer with magnesium chloride (10 x concentration), 0.5 µl *Taq* polymerase solution, 30 µl mineral oil, 1 µl DNA solution.

Two reaction volumes were set up initially, to establish the most appropriate reaction volume as part of the optimisation process:

A separate control tube was set up for each reaction containing all of the above with the exception of the DNA solution which was replaced with water.

The fungal DNA from the wood samples was then amplified using a thermocycler, through a series of predetermined programmes each linked in sequential order:
Program 1: 94°C for 4 minutes (denaturation); **Program 2**: 94°C for 1 minute (denaturation), 36°C for 2 minutes (annealing), 72°C for 1 minute (extension), repeated through 45 cycles; **Program 3**: 72°C for 10 minutes (extension); **Program 4**: samples held at 6°C until ready to be used.

Gel electrophoresis

The amplified DNA samples from the PCR cycle were then 'run' on a 2 % w/v agarose gel using gel electrophoresis. A buffer consisting of 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water was used to load the samples - 5 µl of DNA sample was mixed with 5 µl of loading buffer and pipetted into the wells. A 500 base pair ladder was used as a molecular weight marker (Ampligene, UK). Gels were run at 200 volts / 70 mA for 2 hours, with a running buffer (TBE) containing 2 mM EDTA and 10 mM Tris boric acid. The gels were stained using ethidium bromide solutions (Schlick et al, 1994) and visualised under UV conditions.

Quantification of PCR products

By measuring the intensity of the bands produced using PCR (polymerase chain reaction), the amount of DNA present in a sample can be quantified. This was done using a gel analysis software package from Phoretix International Ltd. (Newcastle-upon-Tyne, UK). The gel was captured as an image using Quantimet Q600 software (Leica Cambridge Ltd., Cambridge, UK and Microsoft) and a colour video camera (3CCD, Sony). The gel image was tensferred to Phoretix 1D Lite and analysed by detecting the bands (these appear as peaks) and measuring the intensity of the peaks.

RESULTS

DNA Concentration

The most suitable amount of DNA to add to the reaction mix from the extracted samples was determined to be 5 µl as this gave the clearest bands in a profile. Optimization of the proportions in the reaction mix is extremely important in order to have excess primer, enzyme and nucleotide. This ensures that the repeated cycling used in PCR will not saturate the enzyme or use up the nucleotides.

Primer Selection

As RAPD primers all have a different sequence and will therefore anneal to a different area of the template DNA (if there is a complementary sequence within the DNA), different sized fragments will result from amplification. Thus different banding patterns are produced by different primers and the most appropriate primer is selected. In this case, RAPD primer OPD-02 was selected on the basis of the distinct banding pattern produced for T60 (see **Fig. 2**).

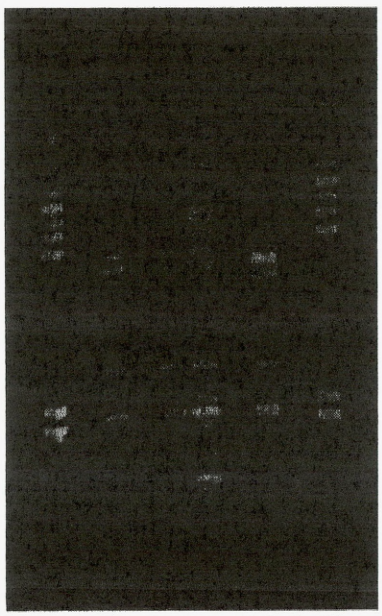
Reaction Volume

Initially a 25 µl reaction was set up at the same time as the 100 µl reactions, but better amplification was observed using the 100 µl volume reactions as the 25 µl reaction did not produce any clear bands. Both of these reaction volumes have been reported as being suitable for PCR (Zimand *et al* (1994) used a 25 µl reaction volume, whereas Theodore *et al* (1995) have successfully used 100 µl reactions) and the decision as to which is the most appropriate is best made by comparing each reaction volume under the individual conditions to be used.

Samples

Figure 3 presents the result of PCR carried out on wood samples from a spruce stake impregnated with T60 spores using the pilot preservation plant. The results demonstrate that PCR is a viable method for determining the distribution of fungal spores in wood for use as a biocontrol agent. The gel shows that fungal DNA is present in samples 1 and 4

(outside samples) and also in sample 6 (an intermediate sample). No T60 spore DNA is present in samples 7 (intermediate) and 2 (from the centre of the stake). This demonstrates that although the spores do not penetrate to the centre of the stake, they can penetrate the wood to a depth of around 10-15 mm, and that this penetration will not necessarily be even and may even depend on the location of an individual stake in the treatment stack.



Lane 1 molecular weight marker
Lane 2 control (no DNA)
Lane 3 T60 control
Lane 4 spruce control
Lane 5 sample 1 (outside)
Lane 6 sample 6 (intermediate)
Lane 7 sample 2 (centre)
Lane 8 sample 4 (ouside)
Lane 9 sample 7 (intermediate)
Lane 10 molecular weight marker
Lanes are numbered 1-10, left to right

Molecular weight markers used - pBr Taq I/Hae III mix, 400ng/5µl (Ampligene, UK)

Fig. 3: Gel showing fungal DNA present in spruce samples from stakes pressure impregnated with fungal spores

Quantification

Quantification of *Trichoderma* (T60) DNA in a particular lane of a gel is represented for T60 control DNA in **Figure 4**. After similar analysis of all lanes in a single gel, intensities of the corresponding bands in different samples can be compared (see **Table 1**). These can be used to correlate DNA intensities in samles from treated wood with those from controls containing DNA from a known concentration of spores. The results from image analysis of the gel show that there is apparently less T60 DNA in sample 1 (outside) than in samples 4 and 6 (outside and intermediate, respectively). This may be due to "washing off" of the spores from the exposed wood surface during treatment - the stake was situated in the pile with both 5 cm faces touching adjacent stakes, and one 2.5 cm face exposed. Any spores penetrating the wood will not be washed off and this may account for the higher proportion of T60 DNA present in sample 6. Sample 4 was from a face not as exposed to the treatment solution as sample 1 and therefore may have been somewhat protected from the washing effects off the treatment solution.

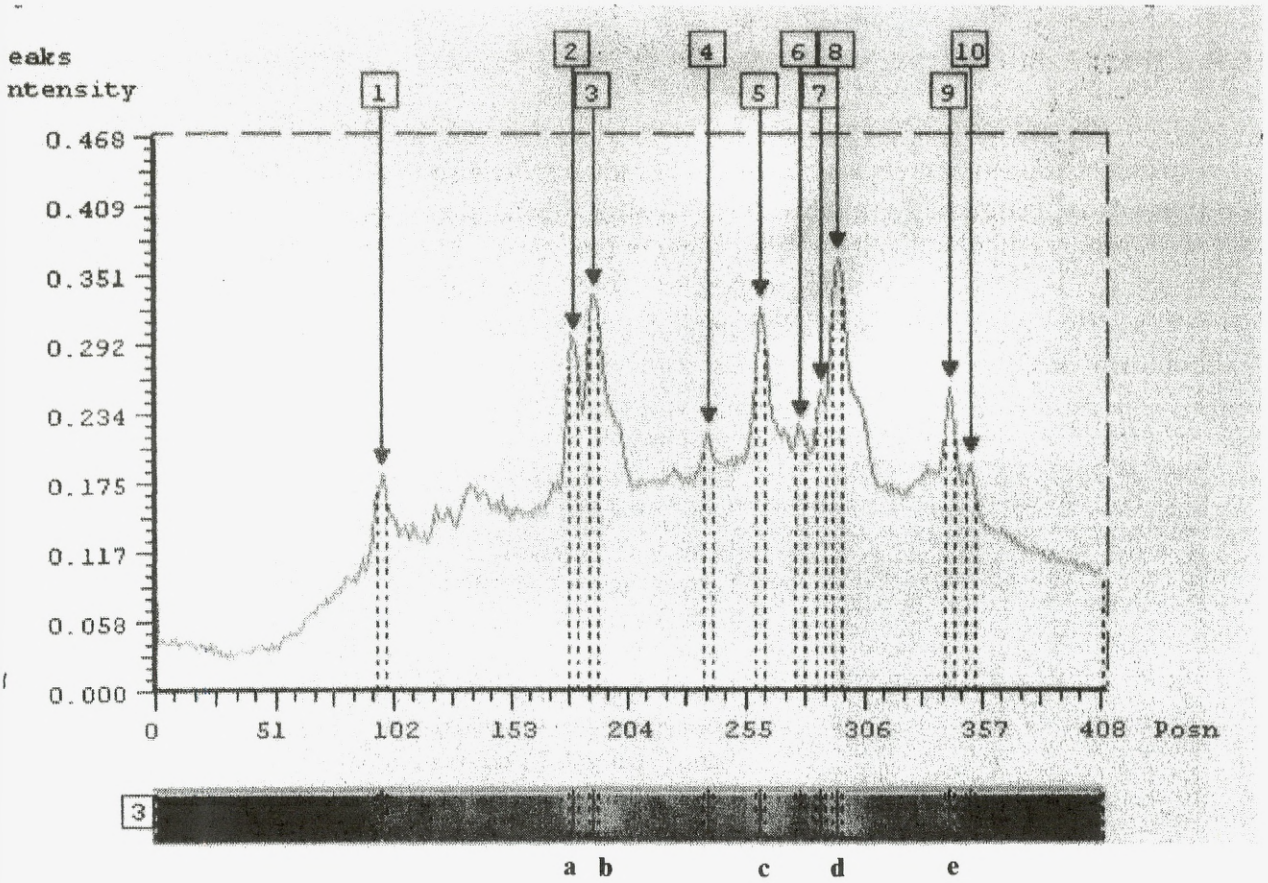


Fig.4: Image analysis printout of a single lane (Lane 3) from fig. 3, depicting peaks (numbered boxes) which correspond to bands of extracted and amplified DNA from T60 spores used as a control. Note peak height represents intensity of the DNA in the band.

	Sample Number (postion in stake)					
Band	**T60**	**1**	**2**	**4**	**6**	**7**
a	1	0.966	0	1.517	1.241	0
b	1	0.818	0	1.812	1.091	0
c	1	1.063	0	1.125	1.250	0
d	1	0.944	0	1.083	1.167	0
e	1	0.840	0	0.880	1.080	0
mean of all bands	1	0.926 (± 0.1)	0	1.283 (± 0.375)	1.166 (± 0.08)	0

Table 1: Ratio of T60 DNA intensity (peak height) in sample bands to that in T60 control bands

DISCUSSION

Many systems can be used to detect and in some instances quantify fungal agents in wood and/or soil. Most however have their individual limitations which reduce their usefulness in certain situations.

An established method of fungal species identification is isolation and growth on selective media followed by morphological and culture comparisons. However, use of selective media and culturing may not provide differential growth of specific isolates from a complex ecosystem such as wood or soil. In addition to this, the extent of mycological expertise in fungal identification that is required provides limitations to anyone lacking experience in mycological taxonomy.

Levels of activity of specific enzymes such as dehydrogenase (Sinclair, 1995) can be used to measure the activity of microorganisms. Dehydrogenase enzymes catalyse the transfer of hydrogen from organic substrates to molecular oxygen to form water. In the dehydrogenase assay, an artificial electron acceptor is reduced by the release of electrons during microbial metabolism, and the reduced form can be measured spectrophotometrically. Measurements of the action of this enzyme in soil do not however give absolute levels of microbial activity and the assay is not selective in that the enzyme activity is not specific to individual fungal or indeed microbial species.

Measurement of fungus specific compounds such as chitin (Chen and Johnson, 1983), glucosamine (Swift, 1973) and ergosterol (Nilsson and Bjurman, 1989; Padgett and Posey, 1993) have also been used to detect and estimate amounts of fungal mycelia in wood and plant tissue and may be considered relatively specific in that they only detect fungi. However, the chitin assay has been criticised for variation in the quantification of chitin content per unit dry weight of mycelium (Nilsson and Bjurman, 1989). The principle disadvantages in the use of signature chemical assays such as ergosterol are that extraction is non-specific (Padgett and Posey, 1993) - the ergosterol extracted from a sample could be from a variety of fungi. The ergosterol content of different species of fungi will vary making quantification difficult, and fungal tissue extracted with ethanol can appear to have a substantially different ergosterol content to that extracted using methanol (Padgett and Posey, 1993). Mycelial age and availability of nutrients will also influence ergosterol synthesis meaning that ergosterol content will be dependant on the substrate in which the fungus is growing. In addition to this, there is a certain amount of ambiguity regarding the exact location of complexed ergosterol within fungal cells, i.e. membrane-bound ergosterol may correlate with fungal mass whereas intracellular fat globules composed of ergosterol esters of fatty acids may interfere with any attempts at correlation against biomass. This means that there can be no direct correlation between ergosterol content and fungal biomass. Therefore, ergosterol cannot be accurately quantified or used to detect and quantify specific strains of fungi.

Molecular detection methods such as protein profiling have been used to distinguish between species but not individual strains or isolates (Palfreyman and Vigrow, 1994).

These authors reported that the majority of *Serpula lacrymans* isolates have identical molecular profiles shown by Western blotting and lectin staining. However, profiles given by old and young mycelia were vastly different, consistent with the production of macro-molecules associated with different growth phase antigens, thereby making specific detection and quantification by this method problematic. Immunological analysis of *Serpula lacrymans* isolates carried out using antisera produced for each isolate did indicate unique antigenic profiles, thus inferring that identification by immunological methods is feasible (Vigrow *et al*, 1991), however antisera produced using mycelial extracts as immunogens were highly cross-reactive. In addition, it was also noted that different morphological forms of the organism and variation in growth medium produced variability in the molecular nature of the major antigens expressed by *S. lacrymans* (Vigrow *et al*, 1991). Although ELISAs using monoclonal antibodies produced against *S. lacrymans* (Burge *et al*, 1994) were more effective in terms of specificity than assays by Vigrow *et al* (1991) using polyclonal antisera, and did not cross-react with other species of fungi, the results of the ELISAs did not demonstrate any ability to distinguish between different isolates of the fungus. There is therefore potential for quantification of fungal species using monoclonal antibody-based assays, but specific quantification of individual isolates using this technology is not possible at present.

PCR is the most appropriate method available for the detection and identification of specific fungal isolates from mixed culture situations. PCR can generate an amplification pattern from small amounts of DNA and theoretically, can therefore be used to detect even single fungal spores in samples. RAPD PCR does not depend on DNA sequence information, unlike standard PCR and therefore can be used when the target DNA sequence is not available. However, there are also limitations with this technology. Equipping a laboratory for PCR work can be expensive, particularly as it is preferable to have dedicated equipment and a separate work area to reduce contamination problems. This is also a practical problem as many institutions will not have the space available for a separate "PCR-only laboratory". In addition to this, optimisation of the process can be initially time-consuming. Aside from the practical problems, PCR can be unsuitable in some circumstances, such as detection or measurement of the activity solely of live microorganisms. PCR will also detect DNA from non-living microbial biomass and therefore DNA detected by PCR is not necessarily that of a live organism, making this technique unsuitable for research designed to assess only viable organisms. Finally, PCR can display hypersensitivity, amplifying unwanted DNA such as that present as a contaminant and therefore great care is required to maintain asepsis throughout the process. However, the disadvantages are far outweighed by the advantages *i.e.* providing a fast, specific detection and identification system for individual fungal isolates. The process can be developed to quantify the PCR products, thus quantifying specific fungal mass in a sample.

Further development

The results presented in this paper are those from the preliminary stages of the esteablishment of a method for the detection and quantification of fungal DNA in wood. As such, the method has not yet been fully developed or indeed validated. It is intended to validate the effectiveness of spore quantification using the Phoretix software by processing non-wood samples containing known quantities of T60 spores before further analysis of the bulk of the wood samples from treated stakes. The reproducibility of PCR and gel electrophoresis for the purposes of quantification using this system has not as yet been established. In addition, the accuracy of quantification may be affected by the lack of adequate facilities for reproducible capturing of gel images to be used for quantitative analysis.

It is intended that the method will be developed further to provide specific detection of individual isolates from within a complex ecosystem i.e. from wood exposed to a wide variety of wood colonising micro-organisms, including several species of *Trichoderma* which may be present in soil. This may be done using the RAPD primers if they prove appropriate (*i.e.* if it can be shown that they can distinguish *T. viride* isolate T60 from other *Trichoderma* isolates), otherwise it is proposed to develop a specific T60 primer using the sequenceof the ITS region. The developed system will then be used to monitor the growth of T60 within wood samples from the field trial stakes currently in place in our field site at Dundee.

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THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

SECTION 1 BIOLOGY

Assessment of the Biocontrol Potential of a *Trichoderma viride* Isolate in a Field Trial

by

Heather L. Brown and Alan Bruce

Scottish Institute for Wood Technology
Department of Molecular and Life Sciences
University of Abertay Dundee
Bell Street, Dundee
DD1 1HG
Scotland, U.K.

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IRG Secretariat
Box 5607
S-114 86 Stockholm
Sweden

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Heather L. Brown and Alan Bruce
Scottish Institute for Wood Technology
School of Molecular and Life Sciences
University of Abertay Dundee, Scotland, UK

ABSTRACT

A field trial has been set up near Dundee, to assess the biological control potential of a *Trichoderma viride* isolate T60. This isolate has been shown in laboratory tests to be particularly effective in protecting wood against certain basidiomycete decay fungi.

Wood was treated with T60 spores using vacuum-pressure impregnation in a pilot preservation plant. Scots pine and Sitka spruce stakes were planted in the field site along with CCA-treated and untreated control samples and also in an accelerated decay facility employed to give a comparison to the field trial results.

This paper presents the results of the first uplift (at 9 months) from both the field and fungal cellar. Preliminary results indicate that there is a noticeable reduction in the rate of sapstain colonisation in T60-treated stakes from the field site, in comparison to untreated stakes. The amount of soft rot decay in stakes treated with a biocontrol agent is significantly lower than that detected in untreated controls from the field site, however this reduction is not apparent in fungal cellar samples.

INTRODUCTION

Wood in ground contact is susceptible to a wide range of wood-decaying micro-organisms. As a result, timber intended for use in ground contact situations is generally treated using toxic chemicals such as CCA, which protect the wood against the effects of biodegradation. However, due to increasing awareness of the environmental impact of wood preservatives, and the introduction of more stringent legislation over preservative disposal and operations at treatment sites, there has, over the last 25 years, been an upsurge of research into the potential of biological control as an alternative technology. During this time, a number of authors (*cited in*: Bruce, 1998) have reported on the uses of biological control agents in agriculture, forestry and forest products.

Many of the reported field trials in biocontrol of wood decay such as those carried out by Bruce *et al* (1986*a*, *b*; 1990) have dealt with remedial treatment of preservative-treated in-service timbers e.g. electricity distribution poles. Although preservatives such as creosote and CCA have been shown repeatedly to prolong the service period of ground contact timbers, premature failure of treated poles has nevertheless been observed. This has often been due primarily to insufficient penetration of the preservative. This can lead to internal decay in the unprotected centre regions of poles.

There have been relatively few field trials investigating the use of biocontrol agents such as *Trichoderma* spp. to protect untreated timber and the majority have been concerned with immediate post-harvest deterioration of timber. Schoeman *et al* (1994) investigated the application of *Trichoderma* spores in chainsaw oil as a means of protecting freshly harvested wood against sapstain and basidiomycete decay organisms. Soil contact is commonly regarded as the most aggressive environment in which wood can be placed. Field trials dealing with the use of biocontrol in non-preservative treated timber for use in ground contact situations are therefore essential to establish the credibility of biological control. Little work has however, been reported on this aspect of biocontrol of wood decay to date.

Trichoderma is currently the most extensively researched biocontrol fungus and has been shown on a number of occasions to have a protective effect against certain wood decay fungi (Bruce and Highley, 1991). Previously reported work (Tucker *et al.*, 1997) has shown that certain isolates of *Trichoderma* have a protective effect against basidiomycete decay fungi. These results, using a modified version of AWPA M10 and EN 113 (1980) as well as a semi-soil burial test system, demonstrated that a *Trichoderma viride* isolate, (T60) was totally effective in protecting wood from the decay action of selected basidiomycetes. As a result, a large-scale experiment designed to assess the biocontrol potential of *Trichoderma viride* isolate T60, has been set up at a field site near Dundee. Although the optimal time period for field trials is a minimum of 5 years, it is valuable to assess results before this and it was therefore decided to employ an accelerated decay facility to provide data from samples for comparison to those from the field.

The use and adaptation of European Standard EN 252 (1989) - "Field test method for determining the relative effectiveness of a wood preservative in ground contact" - will

allow a biological control agent to be assessed using the same parameters as a chemical preservative, *i.e.* to the same point of failure, thereby providing validity for the field trial results when compared with tests of chemical preservatives.

The aim of this paper is to present the results from the analyses of the first uplift of *Trichoderma*-treated field and fungal cellar samples and to discuss the results with regard to the suitability/efficacy of biological control agents for their proposed end use.

METHOD

Although EN252 (1989) contains a brief on the manner in which to carry out a subjective assessment of decay using a scale of one to five, it was decided to supplement this with a series of assessments designed to provide more detailed information about the manner in which the biological agent was affecting the wood and the pattern of colonisation of other micro-organisms. A combination of physical assessments and biological tests were therefore selected. To this end the following parameters were assessed:- sapstain discoloration (visual assessment); soft rot decay (visual assessment, pilodyne and microscopy); basidiomycete decay (visual assessment and isolation); *Trichoderma* colonisation (isolation on selective media); moisture content (electrical conductivity measurements and dry weight analysis).

Wood preparation

The following parameters were selected from the EN 252 (1989) standard: wood type and size, reference preservative and burial pattern. One hundred and twenty Sitka spruce stakes and 120 Scots pine stakes (500 mm x 50 mm x 25 mm) were dried at 40^{o}C for two weeks then allowed to condition at ambient temperature (10-12^{o}C) for 6 weeks, after which their moisture contents were measured using a Diagnostic Timbermaster moisture metre (Protimeter plc, Bucks., UK). The timber was then treated using the pilot preservation plant (see below) and allowed to condition for a further 9 weeks before being planted in either the field or fungal cellar sites. The wood was divided equally into *Trichoderma*-treated, CCA-treated and untreated controls. Forty spruce stakes and 40 pine stakes for each treatment, half of which are situated in a field site at Tealing near Dundee and the remainder in the fungal cellar. The fungal cellar has been maintained at 25 ^{o}C and 80% relative humidity.

Pressure treatment

Preliminary experiments to assess the suitability of pressure impregnation for use with fungal spores and to determine their penetration into wood blocks were undertaken using a bench-top pressure impregnation system as described in AWPA M10 (1977). Blocks treated with a T60 spore suspension were sectioned and slivers of wood from various depths into the block were plated out onto 3% malt extract agar. This established that the spores survived the treatment and that growth was recorded from within the stakes.

Having established the viability of spores following bench-top pressure impregnation, the appropriate pressure treatment cycle for use in the preservation plant (pilot model, Hicksons UK.) was determined by "treating" both spruce and pine stakes using water and the recommended cycles for each wood type. This was undertaken to assess solution uptake and to determine the extent of stake-to-stake variations. The treatment cycle finally selected for both wood types was that recommended for pine to be used in ground contact (BS4072 (1987), BS5589 (1989)): initial vacuum - 600 mmHg for 30 minutes followed by a 90 minute pressure period at 12.8 Kg/cm^2. A spore suspension (8 x 10^5 spores/ml) in around 900 litres of water sterilised using purification tablets (Puritabs Maxi (sodium dichloro-s-triazinetrione 425mg), Schering-Plough Ltd. Mildenhall, Suffolk, UK.), was prepared from approximately 500 plates of T60 grown

on 3% malt extract agar. Stakes were then pressure-impregnated with either fungal spores or a reference chemical preservative (Tanalith CCA - 3%, Hicksons, UK). No final vacuum was drawn on the spore treatment cycle to avoid removal of any spores from the wood surface.

Measurement of results

While the stakes were positioned in the field and fungal cellar, sapstain discoloration was visually assessed and moisture contents were measured using an electrical moisture meter every 4-6 weeks. This was done by selecting "sample" stakes which have been used on every occasion that moisture measurements were taken, to establish a "profile" of the moisture contents in the field trial and accelerated decay facility stakes. Sapstain was recorded for every stake using an arbitrary scale of 0 - 4 based on percentage of stake surface covered with sapstain, and will continue until there is no further change in the degree of discoloration of the stakes.

After 9 months in the field, the first set of stakes was uplifted. Five stakes of each species from each treatment were selected at random i.e. 5 CCA-treated spruce stakes, 5 CCA-treated pine stakes etc. Subjective analysis of stake condition was carried out on site. Pilodyne measurements of surface softness were taken as soon as possible following uplift. Readings were taken from three areas on the front face of the stake, top (4 cm from the end), middle (at the ground line) and bottom (also 4 cm from the end) and additional readings were taken from the same locations on the back of each stake.

Agar plates containing 5% malt extract agar, 4 parts per million benomyl and 0.1% of streptomycin sulphate were used as the selective media for isolating basidiomycetes (Clubbe and Levy, 1977). Slivers of wood were cut from the edges of any suspect (i.e. discoloured (brown or bleached) or softened) patches present on the surface of the stakes. In addition, 8 evenly spaced samples were removed from the area surrounding the groundline. This gave a total of 40+ samples per treatment group. The wood samples were surface sterilised by flaming and placed into the agar. The plates were then incubated at 22°C for 2 weeks and checked daily for signs of basidiomycete growth. Patches of non-basidiomycete growth such as *Mucor* spp. were excised from the plates as soon as possible and any potential basidiomycete growth was subcultured to fresh plates.

At this stage of the methods, 1 stake from each treatment group was removed for whole-stake analysis of moisture content and weight loss. Therefore from this point, all methods were carried out on the remaining 4 stakes from each treatment group.

In order to assess the extent of colonisation of the field trial stakes by *Trichoderma* spp., a *Trichoderma* selective media (TSM) developed by Elad *et al* (1981) was used. Cross-sections measuring 1 cm by 5 cm by 2.5 cm were cut from the top, groundline ("middle") and bottom of each stake. These sections were then cut into smaller samples as illustrated in **Figure 1**.

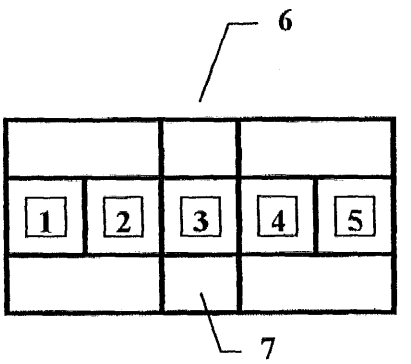

Figure 1: Cross-section showing the position of sample sites within the stakes

A sliver of wood was removed from each section and plated out onto TSM. The plates were incubated at 25°C for 2 weeks and examined for the presence or absence of *Trichoderma* spp. Although growth of contaminants did occur, this was minimal and easily distinguishable from the *Trichoderma* cultures based on their initial morphological appearance.

The blocks of wood (1-7) remaining after removing a sliver for isolation studies were weighed and then oven-dried at 105°C overnight. These blocks were reweighed and the moisture content calculated based on the final dry weight. The resulting moisture contents provided an example of the variation in moisture throughout the stake.

The remaining pieces of the stakes were then assessed for soft rot decay. The three most severe areas of softness were noted and samples were taken for analysis by polarised light microscopy. Sections (1 mm thick) were removed from each site using a scalpel and placed in a test-tube. Two and a half ml of a 50:50 mixture of glacial acetic acid and 100 volume hydrogen peroxide were added and the tubes placed in a boiling water bath to digest the wood. After 90 minutes the samples were removed, covered and vortexed to complete the breakdown of the wood into fibres. A small amount of the digested wood in solution was placed on a glass slide and covered with a cover slip. The samples were then examined using polarised light microscopy to detect the presence of the distinctive diamond-shaped cavities that are characteristic of soft rot decay. Ten fibres were assessed for each sample and given a score on a scale of 0-5 according to the degree of soft rot present where:

0 - no soft rot cavities
1 - 0 - 25 % of the fibre wall covered in cavities
2 - 25 % - 50 %
3 - 50 % - 75 %
4 - 75 % - 99 %
5 - complete degrade of the fibre surface by soft rot cavities

A total soft rot score was then calculated for 10 fibres in each sample and sampling continued at increasing alternate depths of 1 mm until little or no soft rot was detected.

All of the above analyses were carried also out on stakes uplifted from the fungal cellar.

RESULTS

The following key applies to the abbreviations used in tables 1-7:

PTF	pine, *Trichoderma*-treated, field site	**STF**	spruce, *Trichoderma*, field
PPF	pine, CCA-treated, field	**SPF**	spruce, CCA, field
PF	pine, untreated control, field	**SF**	spruce, untreated, field
PTC	pine, *Trichoderma*, fungal cellar	**STC**	spruce, *Trichoderma*, cellar
PPC	pine, CCA, cellar	**SPC**	spruce, CCA, cellar
PC	pine, untreated, cellar	**SC**	spruce, untreated, cellar

Field and Cellar Moisture Contents

The first set of results presents the mean moisture content of the stakes in both the field and the fungal cellar, measured at the groundline region using a moisture metre.

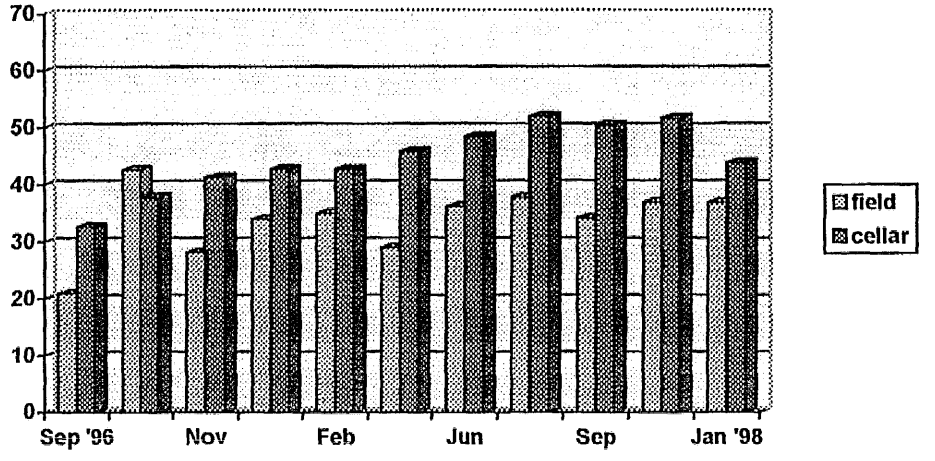

Figure 1: Moisture content (%) in groundline regions of field and fungal cellar stakes.

The results show that within a short time of planting, the field stakes have reached fibre saturation point, and remain at or above that moisture content. The cellar stakes have a higher moisture content overall. Fluctuation in the moisture content of the field stakes does not appear to be seasonal but were associated with individual rainfall events.

Sapstain

The following results (**Table 1**) represent the assessment of sapstain discoloration in field and fungal cellar stakes.

		PTF	PPF	PF	PTC	PPC	PC
mean	**12 weeks**	2	0	3	0	0	0
	22 weeks	3	0	4	0	0	0
	50 weeks	4	0	4	2	0	1
		STF	**SPF**	**SF**	**STC**	**SPC**	**SC**
mean	**12 weeks**	1	0	1	0	0	0
	22 weeks	2	0	2	0	0	0
	50 weeks	4	0	4	1	0	1

Table 1: Visual assessment of sapstain discoloration in stakes.
(0 - no sapstain discoloration; 1 - <25% discoloration; 2 - 25-50% discoloration; 3 - 50-75% discoloration; 4 - >100% discoloration)

No sapstain was recorded in any of the CCA-treated stakes. Of the remaining treatment groups, there was much less sapstain discoloration in the cellar stakes than in the field stakes, for both pine and spruce. The degree of discoloration was higher in the pine stakes than in the spruce, and the onset of sapstain in the untreated pine field samples was faster than in the T60-treated stakes. This effect was not observed in the spruce field stakes or the cellar stakes for either wood species.

Pilodyne Testing

Table 2 presents the results of the pilodyne testing carried out on stakes shortly after uplift. Note the higher recorded readings in the groundline and below groundline regions in comparison to the measurements taken at the top of the stakes.

		PTF	PPF	PF	PTC	PPC	PC
mean (mm)	**top**	6.51 (0.83)	5.60 (0.56)	6.14 (0.94)	6.80 (1.29)	4.75 (0.81)	6.50 (0.63)
	middle	10.05 (1.19)	7.78 (1.27)	9.55 (1.04)	11.05 (2.08)	6.60 (1.22)	13.00 (4.73)
	bottom	10.73 (1.42)	9.14 (1.02)	9.74 (1.10)	11.60 (1.77)	7.35 (1.40)	11.97 (2.36)
		STF	**SPF**	**SF**	**STC**	**SPC**	**SC**
mean (mm)	**top**	9.38 (1.45)	9.43 (2.06)	8.05 (1.37)	11.35 (2.11)	8.45 (1.39)	10.15 (1.98)
	middle	15.34 (2.60)	13.44 (1.63)	13.65 (3.48)	14.90 (1.88)	12.65 (1.86)	15.15 (5.78)
	bottom	16.24 (3.17)	13.87 (1.96)	13.35 (3.03)	15.15 (2.72)	13.05 (1.71)	15.65 (5.07)

Table 2: Resistance to impact measured using a pilodyne. Figures in parentheses represent standard deviations. Values represent the mean of 8 readings.

Through the use of statistical analysis (one-way analysis of variants - ANOVA) it has been possible to determine that there are no significant differences between the pilodyne measurements for T60-treated wood and untreated control stakes for any sample groups. The CCA-treated wood was, as expected, harder than the stakes in other groups.

Decay Fungi Isolation

Basidiomycetes were isolated from 2 stakes in the fungal cellar samples, both untreated controls, one spruce and one pine. Although there were some small bleached and softened patches on one untreated control field sample (comprising about 2% of the total area), isolations were unsuccessful.

Trichoderma **Isolation**

Table 4 presents the results of the isolation of *Trichoderma* on TSM.

	Top							Middle							Bottom						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
PTF	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+
PF	-	-	-	-	-	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+
PPF	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	+	+	-	+	+
PTC	-	-	-	-	-	+	+	-	+	-	-	-	+	+	+	-	-	+	+	+	+
PC	+	+	-	+	+	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+	+
PPC	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-
STF	+	-	-	-	+	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+
SF	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+
SPF	-	-	-	-	-	-	+	+	-	-	-	+	+	+	+	-	-	-	+	+	+
STC	+	-	-	-	+	-	+	+	+	-	-	+	+	-	-	+	-	-	+	+	+
SC	-	+	-	-	+	+	+	+	-	+	-	-	-	+	+	-	-	+	-	+	+
SPC	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-

Table 4: Isolation of *Trichoderma* from field and fungal cellar stakes.
(+/- indicate presence (+) or absence (-) of *Trichoderma* spp. Numbers 1-7 refer to the sample location within the cross-section of wood as shown in **Figure 1**.)

The results show successful isolation of *Trichoderma* in most stakes at the groundline sampling point, particularly in the field samples. There is also evidence of *Trichoderma* in the CCA-treated stakes, but as this appears to be mostly from the outer regions of the stake (1, 5, 6 and 7) it may simply represent surface "contamination". A large proportion of samples from the below ground sampling points also yielded *Trichoderma* growth.

Soft Rot Analysis

The following results (**Table 5**) are those obtained using polarised light microscopy to detect signs of soft rot decay in wood fibres.

		PTF	PPF	PF	PTC	PPC	PC
score (mean)	**1 mm**	7.75 (2.62)	1.58 (3.64)	14.50 (4.21)	42.92 (4.27)	1.67 (1.88)	41.92 (4.87)
	3 mm	2.08 (1.88)	0 (0)	1.50 (1.00)	23.33 (10.25)	0 (0)	20.92 (9.84)
	5 mm	0 (0)	0 (0)	1.00 (1.41)	10.83 (4.65)	0 (0)	11.92 (6.16)
		STF	**SPF**	**SF**	**STC**	**SPC**	**SC**
score (mean)	**1 mm**	6.92 (4.13)	0.25 (0.83)	13.33 (5.54)	34.75 (7.52)	1.02 (1.32)	34.33 (10.32)
	3 mm	1.67 (2.06)	0 (0)	3.58 (3.60)	23.42 (6.39)	0 (0)	17.42 (4.93)
	5 mm	0 (0)	0 (0)	0.92 (1.51)	10.17 (5.37)	0 (0)	7.92 (3.17)

Table 5: Soft rot index based on a mean score out of 50. (Standard deviations on four stakes are presented in parenthesis).

No significant soft rot was found in any of the CCA-treated stakes, and the results also demonstrate that the amount of soft rot present in the *Trichoderma*-treated field stakes is around half that in the corresponding controls. This effect is not, however, apparent in the fungal cellar samples where the amounts of soft rot are similar in the T60-treated and untreated stakes. Soft rot is more severe in the cellar samples than in the field stakes and is also deeper into the wood, as can be seen in the table by comparing values for PC against PF at all depths.

Final Stake Moisture Contents

Table 6 represents the mean moisture contents of the samples measured by dry weight analysis. It can be seen that within any one treatment group, standard deviations are high. This is primarily due to intra-stake variations.

		PTF	PPF	PF	PTC	PPC	PC
mean	**top**	18.07 (0.76)	15.00 (0.62)	16.42 (1.01)	45.26 (27.45)	23.06 (0.93)	22.58 (1.25)
	middle	56.22 (24.07)	23.18 (3.56)	41.27 (10.58)	114.32 (0.05)	28.63 (0.85)	35.95 (4.40)
	bottom	66.48 (20.04)	40.03 (21.81)	56.07 (15.24)	108.76 (14.37)	39.58 (9.78)	34.31 (5.88)
		STF	**SPF**	**SF**	**STC**	**SPC**	**SC**
mean	**top**	13.93 (0.72)	16.66 (0.59)	14.17 (0.68)	42.98 (22.15)	27.00 (0.79)	24.61 (0.84)
	middle	24.87 (12.28)	53.78 (19.12)	36.89 (15.37)	92.78 (52.00)	40.99 (6.31)	34.95 (4.25)
	bottom	114.97 (50.5)	111.75 (39.00)	40.04 (33.51)	157.28 (61.62)	80.38 (34.17)	45.63 (16.15)

Table 6: Mean moisture contents (%) for each treatment group measured by dry weight analysis. Figures in parentheses represent standard deviations and each value represents a mean of 28 readings.

The results demonstrate a clear top-to-bottom moisture gradient within the stakes. *Trichoderma*-treatment of stakes has had the effect of increasing moisture content, especially noticeable in the cellar stakes where groundline moisture contents are around 100 %. It is possible that the lower moisture content in the field and cellar pine stakes is attributable to the effects of CCA on moisture uptake.

The following results (**Table** 7) provide an example of the moisture contents of individual stakes.

	M. C. %	
Block No.	**PTF 9**	**PTC 3**
Top 1	18.65	31.43
T2	18.56	39.30
T3	18.52	55.32
T4	19.13	60.30
T5	18.59	59.86
T6	18.03	88.80
T7	18.22	52.37
Middle 1	51.82	143.51
M2	35.45	94.32
M3	36.91	96.82
M4	25.48	90.29
M5	47.62	119.40
M6	47.65	118.70
M7	54.31	137.50
Bottom 1	55.09	118.85
B2	74.89	84.02
B3	45.28	78.92
B4	46.50	76.66
B5	57.42	107.24
B6	48.55	91.32
B7	51.46	103.72

Table 7: Inter- and intra-stake variations in moisture content (%) measured by dry weight analysis. (Note that samples 1, 5, 6 and 7 represent surface samples.)

It is clear from these results that moisture contents are higher at the surface of the stakes (samples 1, 5, 6 and 7) than in the stake interiors (samples 2, 3 and 4). Note the increase in variability in the cellar sample (PTC3) in comparison to the field sample.

DISCUSSION

The results presented in this paper represent only the first uplift from both the field site and the fungal cellar, and a clearer indication of the success or failure of the system will be produced once all samples have been uplifted. It is nevertheless already apparent that there is a definite effect of the pre-treatment with the biocontrol agent on both the patterns and extent of decay in the stakes.

In order for biological control to be considered a viable option in timber preservation, it is necessary to investigate additional aspects such as formulation; efficacy of the delivery systems; and the influence of the organism on the microbial succession pattern within the test system as well as the protective effect. Traditional chemical treatments employ vacuum-pressure impregnation as a means of ensuring the maximum possible penetration and retention of chemical formulations in timber. For biological control to become an acceptable alternative to timber preservation, it would be advantageous if delivery of biocontrol agents could follow standard industrial procedures as closely as possible. The use of fungal spores as biocontrol agents has already been investigated in the remedial treatment of *Serpula lacrymans* by spraying infected wood with a *Trichoderma harzianum* spore suspension (Score, 1998), and in the biocontrol of basidiomycete decay fungi, by treating wood blocks with a *Trichoderma* spore suspension using a bench-top pressure impregnation system (Tucker *et al.*, 1997). This study establishes, however, that a pilot preservation plant can be used to pressure impregnate wood with a fungal spore suspension for biocontrol purposes. Spores retained viability following pressure treatment in this study, but the exact nature of the uptake and penetration of spores into the wood was not determined. A molecular detection system based on the polymerase chain reaction (PCR) is therefore being developed to detect and quantify spores through the use of image analysis (Brown and Bruce, 1997). This system will be used in future uplifts to determine the efficacy of pressure impregnation of fungal spores in spruce and pine stakes.

Trichoderma spp. were re-isolated from a majority of the pre-treated stakes, primarily from the groundline and below groundline sampling points. *Trichoderma* spp. were also isolated from the untreated control samples, however this is to be expected as *Trichoderma* isolates are a naturally-occurring soil organisms and the isolation methods used do not differentiate between individual isolates. The results of the TSM isolation plating also revealed the presence of *Trichoderma* in CCA-treated stakes, although this is limited largely to outside edges where it is possible for the fungus to enter the stake but not colonise to any significant depth. In out-of-ground contact areas such as the top sampling points, *Trichoderma* was not isolated with as high a frequency as in the ground contact areas. This may indicate that spore germination/viability is poorer in those areas of the stakes which tended to remain drier.

Basidiomycete colonisation and decay was assessed using isolation plating, but initial observations were also made regarding the percentage of the stake surface which was covered with decay pockets. These observations showed that there was very little colonisation by basidiomycete fungi in the field trial stakes, and decay fungi were only present in the untreated wooden stakes. In the cellar samples, there was more

basidiomycete colonisation as evidenced by decay patches, including stake PC4 which revealed significant internal decay by brown rot fungi when cross-sectioned for sampling. These results are in direct correlation with the findings of Tucker *et al* (1997), where pre-treatment of wood blocks with *Trichoderma* spores resulted in no significant decay when the wood was exposed to basidiomycete decay fungi.

A significant reduction in the amount of soft rot was also noted in the T60-treated stakes in the field samples. This correlates well with a previous lab-based study carried out using non-sterile soil, designed to assess the effectiveness of the potential biocontrol agents against a wider range of wood degrading micro-organisms (Brown *et al*, 1996). Although there was measurable decay in all of the blocks, there was a significant reduction in the rate of decay in the *Trichoderma*-treated blocks. Soft rot in the cellar samples was, however, more severe and similar in both the T60-treated and untreated stakes. This may indicate that the biocontrol organism selected is more effective under field conditions, as opposed to the controlled environment of the fungal cellar or that different populations of organisms are responsible for the soft rot in the two systems and the biocontrol is more successful against the field soft rot species. It may however simply be that the acceleration of the decay process in the fungal cellar has masked any initial protective effect by the *Trichoderma*. Extended field testing will indicate if the reduced rate of soft rot due to the *Trichoderma* is maintained at subsequent uplifts.

Pre-treatment with *Trichoderma* spores had a marked effect on the rate of sapstain colonisation of Scots pine stakes in the field, when compared to the rate at which discoloration appeared in the corresponding controls. This effect was not apparent in the T60-treated Sitka spruce stakes in which the rate of sapstain development was similar to that in the untreated spruce controls. Pine has a higher proportion of soluble nutrients than spruce (Nayagam, 1987) and this may explain the increased rate of sapstain colonisation observed in the pine stakes. The observed biocontrol in the *Trichoderma*-treated pine stakes may be a result of competition for those nutrients. Alternatively, lack of protection in the spruce stakes may simply indicate a lower level of spore treatment and subsequently lower colonisation of the spruce by the biocontrol agent due to the refractory nature of the timber, and lower nutrient availability.

Although the results of the pilodyne testing do not appear to show any major differences in the resistance to impact between the treatment groups, there are several noticeable differences when the results were analysed using Dunnets' test. In all groups except the field spruce samples, there were significant differences between the *Trichoderma*-treated stakes and the CCA-treated stakes, and between most of the untreated samples and the CCA-treated controls (see **Table 3**). The resistance to impact of the CCA-treated wood was lower than in other treatment groups. This is expected as the use of CCA can increase the hardness of wood (Jonnsson *et al*, 1989). This is thought to be due to the formation of rigid polymeric structures during chromium (VI) fixation with lignin and cellulose (Hainey, 1992; Pizzi, 1979; 1981). No significant differences were found, however, between the untreated stakes and the *Trichoderma*-treated stakes. A number of reasons may account for differences in pilodyne recorded values, including natural variations in the density of the wood (i.e. between growth rings); variations in moisture content; and the effects of decay. However, in most of the untreated control stakes and some of the *Trichoderma*-treated

stakes, moisture does not appear to be a factor in the decreased resistance to impact of the wood (i.e. there is no correlation between increases in moisture content and pilodyne measurements, suggesting that this is instead due to decay, particularly in the cellar samples (see **Table 6**).

The moisture contents presented demonstrate a high variability, due in part to the sampling points selected. However, another factor which may account for this variation, particularly in the cellar samples, is variations between the different soil beds. CCA-treated stakes have been reported previously to have a lower moisture content than untreated wood (Green, 1988) and this can be clearly seen in the pine stakes even in the cellar samples (see **Table 6**). In addition, pre-treatment with *Trichoderma* spores appears to have had a marked effect on the moisture content of the stakes, with the moisture contents of T60-treated wood higher than the corresponding controls. This may be a result of the cellulolytic and pectinolytic action of *Trichoderma* spp. enzymes, degrading pit membranes and increasing the permeability and moisture-transfer capabilities of the wood (Johnson and Gjovik, 1970).

Conclusions

Overall, the results demonstrate that there is a significant effect in the field samples of reduced colonisation and decay of stakes treated with T60 spores. The rate of development of sapstain discoloration in the pine stakes was also noticeably reduced, and the degree of soft rot present in the T60-treated field stakes was found to be around half that found in the untreated controls. Although these findings were not repeated in the cellar samples, they have provided some valuable information regarding moisture uptake and its effect on the potential biocontrol isolate. The conclusions drawn from these results are that, as with previous laboratory testing (Tucker et al, 1997; Brown et al, 1996), there is a reduction in the rate of both soft rot and basidiomycete decay in wood pre-treated with *Trichoderma* spores but complete, long-term protection has not been achieved especially with regard to soft rot.

Future work in this project will focus on the remainder of the field and fungal cellar uplifts, along with the development of the PCR detection system to provide a more complete picture of *Trichoderma* colonisation and decay protection.

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Assessment of the biocontrol potential of a *Trichoderma viride* isolate. I. Establishment of field and fungal cellar trials.

Brown, H. L. and Bruce, A. (1999)

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